~~The identification of antibiotic-resistant bacteria and antibiotic resistance genes from the culturable oral microbiota~~

DETECTION AND CHARACTERISATION OF GENES ENCODING ANTIBIOTIC RESISTANCE IN THE CULTIVABLE ORAL MICROFLORA.

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Abstract

The emergence of antibiotic-resistant bacteria has become a major threat to public health. The increased use of antibiotics has selected for the dissemination of antibiotic resistance genes between organisms from different species and different genera. There is a large body of evidence that the indigenous microbiota can act as a reservoir of antibiotic-resistant bacteria. However little is known about the molecular basis for this in bacteria from the oral cavity. Therefore the aim of this work was to determine the prevalence of antibiotic-resistant bacteria and antibiotic resistance genes in the cultivable oral microbiota. Saliva and plaque samples were taken from each of 60 healthy adults who had not taken any antibiotics during the previous three months. Each sample was plated onto antibiotic-containing media to quantitate and identify antibiotic-resistant strains. All of the individuals harboured bacteria resistant to erythromycin, gentamicin, vancomycin and tetracycline. Only 4 individuals (7%) did not have any cultivable bacteria resistant to amoxycillin. Oral bacteria resistant to gentamicin were the most commonly isolated (constituting 23% of total cultivable oral bacteria) followed by erythromycin (18% of the total viable count), vancomycin (16% of the total viable count), tetracycline (10% of the total viable count) and amoxycillin (4% of the total viable count). Multiply-resistant bacteria were found with 55% of tetracycline-resistant isolates being resistant also to erythromycin and 6% resistant also to both amoxycillin and erythromycin. The most prevalent genes encoding tetracycline and erythromycin resistance were *tet*(M), *tet*(W), *tet*(O), and *mef* and *erm*(B) respectively. In some cases, *tet*(M) and *ermB* were contained within a Tn*1545*-like conjugative transposon and could be co-tranferred to *Enterococcus faecalis*. Finally the nature of the genetic support for one of the *tet*(W) genes, was determined and found to be flanked by two transposases belonging to two different families of insertion sequences (IS*30* and IS*256*). This element was highly unstable in *E. coli*. This study showed that antibiotic-resistant bacteria and antibiotic resistance genes are present in the oral microbiota and that oral bacteria are likely to play an important role in the evolution and dissemination of antibiotic resistance genes.

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Contents

Tables

Figures

Chapter 1

Chapter 3

Chapter 6

Chapter 7

Abbreviations

ABC	ATP-Binding Cassette Protein
ATP	Adenosine Tri-Phosphate
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
bp	base pair
°C	Degree Celsius
Chr	Chromosome
CNS	Coagulase Negative Staphylococci
cTn	Conjugative transposon
dH_2O	Distilled water
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic Acid
ery	erythromycin
F	F plasmid
g	Gravitational force
GTP	Guanosine Triphosohate
h	Hour
HGT	Horizontal Gene Transfer
ICU	Intensive Care Unit
IS	Insertion sequence
IRs	Inverted repeats
kb	Kilobase
kbp	Kilobase pair
µl	Microlitre
LB	Luria Bertani
µm	Micrometer
M	Molar
MDR	Multidrug Resistance
MFS	Major facilitator super family
min	Minute

ml	Millilitre
MLS	Macrolide, Lincosamide and Streptogramin antibiotics
mm	millimetre
mM	Micro-molar
MRSA	Methicillin resistant *Staphylococcus aureus*
NBU	Non-replicating Bacteroides Units
Neiss	*Neisseria* spp.
nm	nanometer
orf	open reading frame
*Ori*T	Origin of Transfer
PBPs	Penicillin Binding Proteins
PCR	Polymerase Chain Reaction
PRP	Penicillin-Resistant *Streptococcus pneumoniae*
QAC	Quaternary Ammonium Compounds
RBS	Ribosome Binding Site
Ref	Reference
rev	revolution
RNA	Ribonucleic Acid
RND	Resistance Nodulation Division
rpm	revolutions per minute
RPP	Ribosomal Protective Proteins
sec	second
SDS	Sodium Dodecyl Sulphate
SMR	Small Multidrug Resistance protein family
SSC	Standard Saline Citrate
Staph	*Staphylococcus* spp.
Strep	*Streptococcus* spp.
tet	tetracycline
TBE	Tris Borate EDTA
Tn	Transposon
VGS	Viridans Group Streptococci
VRE	Vancomycin-Resistant Enterococci

Chapter 1

Introduction

1.1 The evolution of bacteria and infectious diseases

Bacteria existed long before the evolution of humans (208); they were, and still are, responsible for various infectious diseases such as tuberculosis, plague, cholera; some of which were shown to be common in prehistoric humans (316). However the mechanism of pathogenicity of bacteria became apparent only with the discovery of this microworld, and it led to the discovery of antibiotics to treat these infections. From then on a new fascinating world was revealed; bacteria were shown to inhabit different environments and be able to transfer information between one another so as to facilitate their adaptation and survival (240). However one of the consequences of this adaptation was the emergence of antibiotic-resistant bacteria, which have now become a major threat to public health (122).

1.1.1 The relationship between humans and bacteria

1.1.1.1 History

The History of antimicrobial chemotherapy

- *2000 B.C. – Here, eat this root*
- *1000 A.D. – That root is heathen. Here, say this prayer.*
- *1850 A.D. – That prayer is superstition. Here, drink this potion.*
- *1920 A.D. – That potion is snake oil. Here, swallow this pill.*
- *1945 A.D. – That pill is ineffective. Here, take this penicillin.*
- *1955 A.D. – Oops....bugs mutated. Here, take this tetracycline.*
- *1960-1999 – 39 more "oops"...Here, take this more powerful antibiotic.*
- *2000 A.D. – The bugs have won! Here, eat this root.*

— Anonymous from the WHO report.

Over time a pattern emerged between the evolution of bacteria and the evolution of civilisation. In the early days humans were living as nomads rather than in large groups, which limited the spread of disease. No domestic animals were kept, curtailing any spread of diseases by reducing the contact between humans and animals. But then humans started living in societies, in villages raising animals and crops for food. People and animals often shared the same dwelling, and as a result of this close proximity infectious diseases emerged (137). The appearance of epidemics is a good indicator of the ready adaptation of bacteria to new environments. Movement of people into larger settlements or military invasion encouraged these epidemics, affecting only local people, to spread, sometimes into pandemics affecting the whole population or a number of countries (Table 1.1).

Table 1-1: Bacterial infections and humans

Period	Infection	Infectious agent	History
430 BC-1542	The plague	*Yersinia pestis*	Spread across Egypt, Rome, Syria, China and Europe killing millions of people and sometimes marked a turning point in the history of a population.
1492	Tuberculosis and gonorrhoea	*Mycobacterium tuberculosis* and *Neisseria gonorrhoeae*	These already endemic european diseases spread to the Caribbean with Columbus leading to the infection of millions of local people who had no immunity to them.
Early trading period and the 16th century	Dysentery	Enteric pathogens	Caused devastating epidemics with African and European infections. It was then introduced by the Spanish into Central and South America.
As trade journeys lengthened	Chronic infections such as tuberculosis	*Mycobacterium tuberculosis*	Introduced by European sailors to the Pacific Islands.
Present time	Lyme disease, chlamydia, gonorrhoea, tuberculosis, food-borne diseases	*Borrelia burgdorfer, Chlamydia trachomatis,*	Even during the past few decades, there has been a resurgence of epidemics (38, 107, 227).

Adapted from Kiple KF (137).

1.1.1.2 Discovery of the microworld

The first description of bacteria was made by van Leeuwenhoek who observed them through a microscope at the end of the 17th century (290). Then in 1857 Louis Pasteur noted that some microorganisms (defined as organisms that are so small that they are invisible to the naked eye) could be responsible for fermentation and putrefaction leading him to invent pasteurization in 1863, a process by which fermentation and thus contamination is prevented by sterilising a medium (http://www.pasteur.fr/externe.html). Ten years later Robert Koch discovered the cause of anthrax in horses by isolating anthrax bacilli and showing their abiliy to cause diseases and their capacity to form spores (186). He deduced that specific organisms could produce specific diseases; he went on to develop techniques to culture bacteria in the laboratory that resulted in a Nobel Prize in 1905 (186). At the end of the 19th century, Vuillemin noticed that fungi could destroy bacteria; this was further investigated by Alexander Fleming, who reported in 1929 the antibacterial action of a fungus, *Penicillium notatum* (89). However it was only in 1940 that the role of penicillin G was recognised and used to treat various bacterial infections (41). The age of antibiotics had begun; some bacteria were found to be resistant to penicillin (1) and so the search for new antibiotics started and it continues to this day.

1.1.1.3 Increase in antibiotics and antibiotic-resistant bacteria

Anti-infective agents have been used for thousands of years to treat infectious diseases; sulfur and mercury were used to treat scabies and syphilis respectively (286). However the search for satisfactory antibacterial therapy was unsuccessful until the early 20th century when sulphonamides were discovered. In 1927, the German scientist Gerhard Domagk discovered sulphonamides (Table 1.2) and received the Nobel Prize for his work in 1939 (286). He used sulphonamide, then known as protonsil red, to relieve his daughter of a persistent streptococcal infection (75). Further studies identified the sulphonamide group as the active ingredient; sulfanilamide derivatives proved effective against pneumonia, meningitis, blood poisoning and gonorrhoea (286). This was followed by the discovery of penicillin in the mid 20th century (1). This discovery triggered a health care revolution, and from

this flowered an entire family of penicillin-based antibiotics (178). Selman Waksman isolated the first aminoglycoside antibiotic in 1944 from an *Actinomyces, Streptomyces griseus* (63), and Benjamin Duggar discovered the first tetracycline class of antibiotics in 1949 by isolating chlortetracycline and oxytetracycline from *Streptomyces aureofaciens* and *Streptomyces rimosus* respectively (45). Macrolide antibiotics were discovered by McGuire and colleagues in 1952 in *Streptomyces erythreus* (247). Among other important antibiotics discovered was the glycopeptide vancomycin in 1956, the product of *Streptomyces orientalis* (149). After a flurry of discoveries between 1930 and 1970, the past 40 years have witnessed fewer discoveries (325). Although antibiotic resistance has been a problem since the 1940s, new antibiotics have allowed physicians to keep ahead of resistant strains. However we are now experiencing a reduced availability of new antibiotics to curtail infections caused by resistant organisms.

The introduction of a new antibiotic was often followed by the development of resistance in bacteria (Table 1.2). The first penicillin-resistant *Escherichia coli* appeared in the 1940s (1) and were soon followed by penicillin-resistant *Staphylococcus aureus*, and in 1967 penicillin-resistant *Streptococcus pneumoniae* and *Neisseria gonorrhoeae* (Table 1.2). Streptomycin-resistant enterococci appeared in 1967 in Japan (63). Erythromycin was used in 1952 as the first macrolide antibiotic and within a year erythromycin-resistance appeared in *Staphylococcus* spp. in the US, Europe and Japan (247). Prior to the mid-1950s, the majority of bacteria were susceptible to tetracycline (123), the first tetracycline-resistant bacterium, *Shigella dysenteriae*, was isolated in 1953, followed by a dramatic increase in tetracycline-resistance in pathogenic as well as commensal bacteria (45). This direct correlation over time between use of antibiotics and the increased proportion of resistant to non-resistant strains indicated that bacteria were evolving in response to the selective pressure due to increased use of antibiotics.

Table 1.2: Emergence of resistance to newly-discovered antibiotics

Antibiotic	Year discovered	First resistant strain	Reference
Sulphonamide	1927	1946, *Streptococcus* spp.	62
Penicillin	1929 (but used commercially in 1940)	early 1940s, *E. coli*, *S. aureus*, 1967, *S. pneumoniae*, *N. gonorrhoeae*	1 106
Aminoglycoside	1944	1967, *Enterococcus* spp.	63
Chloramphenicol	1947	1950, *Salmonella typhi*	55
Tetracycline	1949	1953, *Shigella* sp.	45
Macrolide	1952	1952-53, *Staphylococcus* spp.	247
Vancomycin	1956	1987, *Enterococcus* spp.	149

1.1.2 Antibiotic-resistant bacteria: they are everywhere!

The wide use of antimicrobial agents in human medicine and agriculture (Table 1.3), has led to the emergence of infections caused by bacteria that have become resistant to most available drugs so that antibiotic-resistant bacteria have become a major threat to public health (122).

Table 1.3: Use of antibiotics

Where antibiotics are used	**Types of use**	**Questionable use**
Human use (50%) [a]	20% Hospital 80% Community	20-50% Unnecessary [b]
Agricultural use (50%) [a]	20% Therapeutic 80% Prophylactic/growth promotion	40-80% Highly questionable

Table taken from Wise *et al.* (328).

[a] 18 million kilograms of antibiotics are used in the US each year (309).

[b] Prophylactic use of antibiotics before surgery, empiric use (without knowing the aetiological agent), paediatric use for viral infections are some of the factors that have led to an overuse and misuse of antimicrobial agents in humans (121).

1.1.2.1 In the hospitals

Hospitals offer a prime opportunity for the development and transfer of antibiotic resistance, because of heavy exposure to antimicrobial agents, patients prone to infections and a high population density (296). Consequently they now have to face the emergence of nosocomial infections caused by multidrug-resistant Gram-positive and Gram-negative bacteria.

1.1.2.1.1 MRSA (methicillin-resistant *Staphylococcus aureus*)

MRSA is a major cause of hospital-acquired infections worldwide (161). These resistant organisms cause bacteraemia, pneumonia and surgical wound infections; they can develop resistance very readily both by mutation and by DNA transfer. The problem started with the appearance of penicillin-resistant *Staphylococcus aureus* soon after the introduction of the antibiotics in the 1940s (Table 1.2); by the 1950s, half of the strains were penicillin-resistant worldwide through the production of β-lactamases, and by the 1980s the figure went up to 90% (161). One response to this has been the development of new β-lactamase-resistant penicillins, such as oxacillin and methicillin. Unfortunately methicillin-resistant *Staphylococcus aureus* emerged in the 1970s and increased in frequency as hospital pathogens during the 1980s and 1990s in many countries (229). Resistance to methicillin is due to the production of altered penicillin binding proteins (PBPs) with low affinity for all β-lactam antibiotics (161). These altered PBPs are encoded by the *mec* gene, carried by large mobile genetic elements inserted into the host DNA and termed staphylococcal cassettes chromosome mec (SCC*mec*), these cassettes consist of the *mec* gene complex (the *mec*A gene and its regulators) and the *ccr* gene complex which encodes site specific recombinases responsible for the mobility of SCC*mec* (135). Most MRSA strains are resistant to most other antibiotics leaving the glycopeptide antibiotics as the last choice to treat such infections (295). Recently, treatment failures caused by some strains with decreased susceptibility to vancomycin were reported in Japan (117), USA (118) and UK (331). The mechanisms of resistance are thought to be due to elevated mutation frequencies allowing the strain to develop resistance by stepwise selection in the presence of increasing concentrations of vancomycin (261). However, recently one *vanA* gene was identified in a strain from the US. This gene is plasmid-mediated and is thought to have been transferred from another nosocomial pathogen, a vancomycin- resistant enterococcus VRE (295).

1.1.2.1.2 VRE (vancomycin-resistant enterococci)

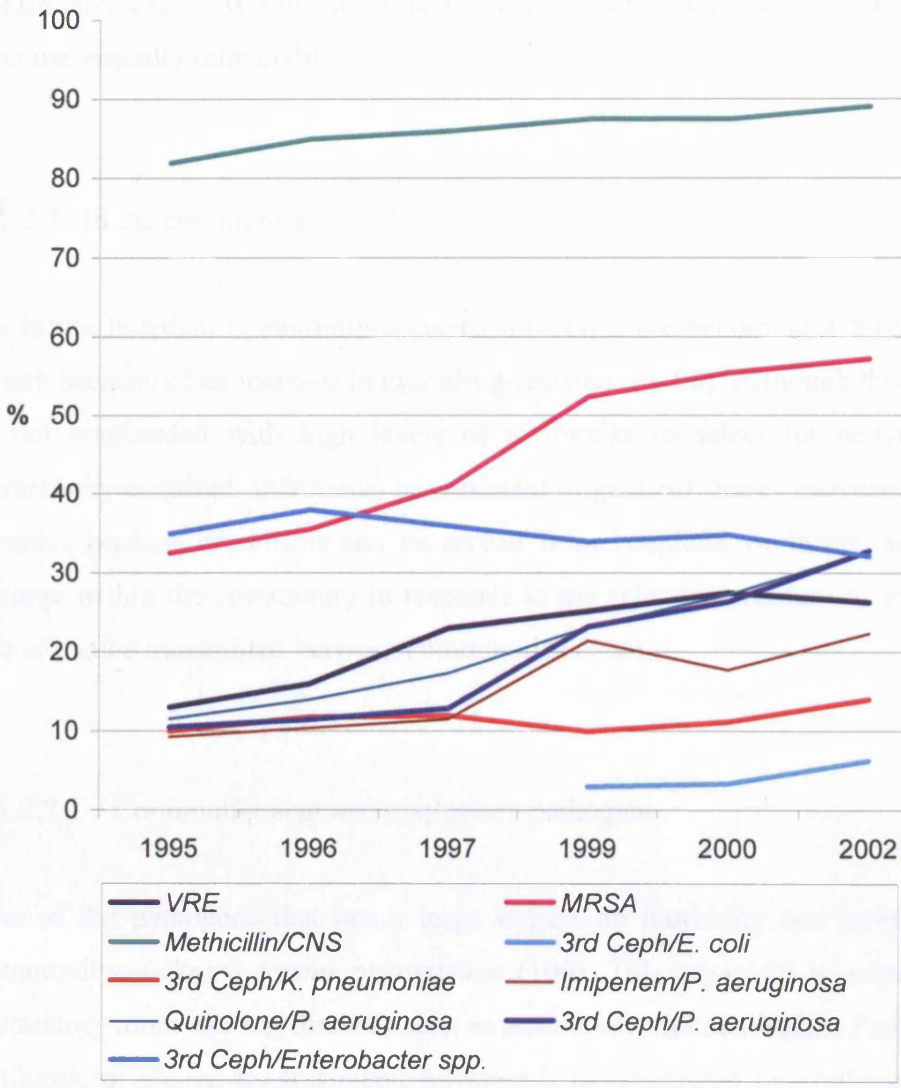
A similar problem with nosocomial infections has been the emergence of VRE (26). Enterococci are normal inhabitants of the gastrointestinal tract. However they have

become a major nosocomial pathogen in the last decade (26). Enterococci are intrinsically resistant to several commonly used antibiotics and they are able to acquire resistance to all currently available antibiotics either by mutation or by transfer of mobile genetic elements (26, 239). The first VRE was reported in the UK and France in 1987 (149) and by 1993 14% of patients with enterococcus infections in intensive care units had vancomycin-resistant strains, the prevalence of VRE in Europe has been increasing ever since (40, 332). The spread has been even greater in the last decade in US hospitals where the figures reached 25% in 2000 (26). The emergence of VRE is thought to have been caused by excessive antibiotic usage and also by cross-contamination in hospitals (27). VanA is the most common mediator of resistance to vancomycin in enterococci in Europe and America (26). The gene is located on a transposon, Tn*1546,* in a cluster of 4 other *van* genes (*vanR*, *vanS*, *vanH*, *vanX*) necessary for glycopeptide resistance (332). This cluster of genes is induced by the presence of the antimicrobial agent which leads to the production of modified target sites with low affinity for glycopeptide antibiotics (332). Recently there has been some controversy about the transmission of VRE from animals to humans (129, 306, 318) as will be discussed later (see chapter 1.1.2.3.1).

1.1.2.1.3 Nosocomial infections due to Gram-negative bacteria

The distribution of antimicrobial resistant pathogens causing nosocomial infections varies with time and among hospitals. However, a common fact observed worldwide is the continuing increase in the prevalence of resistance (Fig 1.1). Multiple antibiotic resistance to useful classes of antibiotics has gradually increased among a number of Gram-negative hospital pathogens creating a wide range of nosocomial infections including bacteraemia, pneumonia, urinary tract and surgical wound infections (109, 229). The increase is particularly marked for third generation cephalosporin-resistant *Enterobacteriacae*, imipenem- and ciprofloxacin-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumanii* because it is worldwide and can compromise the treatment of infections caused by any of these organisms (Fig 1.1).

Figure 1.1: Increase in selected antimicrobial resistant pathogens associated with nosocomial infections in ICU patients in the US from 1995 to 2002



These data were adapted from the results obtained in different studies in the US from 1995 to 2002 (94, 95, 200, 201, 202).

ICU = Intensive care unit, VRE = vancomycin resistant enterococci, CNS = coagulase negative staphylococci, 3rd ceph = resistance to third generation of cephalosporins (cefriataxone, cefotaxime or ceftazidime), quinolone = resistance to either ciprofloxacin or ofloxacin. Resistant rates (%) for each organism from ICU patients from 1995-2002.

In the industrialised world, as many as 60% of hospital-acquired infections are caused by VRE and MRSA. These organisms have now started spreading to the community (325). We are heading towards a situation in which infections might become virtually untreatable.

1.1.2.2 In the community

As in the hospital, community-acquired infections are becoming a threat to public health because of an increase in multidrug-resistance (106). Although the community is not bombarded with high levels of antibiotics to select for resistant strains, community-acquired infections by resistant organisms have increased in recent decades because organisms can be spread from hospitals, or farms, and also can emerge within the community in response to the selective pressure of antimicrobial use or can be transmitted between communities (106).

1.1.2.2.1 Community acquired respiratory pathogens

One of the pathogens that has a large impact on morbidity and mortality in the community is *Streptococcus pneumoniae* (106). This organism is acquired by the respiratory route causing diseases such as pneumonia and meningitis. Penicillin is the antibiotic of choice for treatment; however it is substituted by erythromycin when patients are allergic to penicillin. There have been concerns about the increased prevalence of penicillin- and erythromycin-resistant *S. pneumoniae* as well as multidrug resistant strains. The prevalence of penicillin-resistant *S. pneumoniae* (PRP) is variable in different countries but high prevalence rates have been reported in Spain (84, 224), France (224) and United States, 33.5% (297). Many of these PRP are also resistant to other antimicrobial agents, including tetracycline, chloramphenicol and trimethoprim-sulfamethoxazole (73, 297). *S. pneumoniae* became resistant to penicillin due to the acquisition of mosaic genes (by transformation) that encode for altered PBPs (64, 232). An increased consumption of macrolides was paralleled by an increase in resistance to erythromycin through the acquisition of methylase (*erm*(B)) or efflux (*mef)* genes (83, 98, 234). The prevalence

and the mechanism of resistance to macrolides vary geographically and are likely to be due to differential antibiotic consumption (218).

The emergence of resistance in two other important respiratory pathogens, Group A streptococci (*S. pyogenes*) and *Neisseria meningitidis* has also been observed. The drug of choice for both pathogens is penicillin (106). Whereas *S. pyogenes* is still susceptible to this drug, high rate resistance to erythromycin has been reported; it is due to the acquisition of a methylase or efflux genes as in *S. pneumoniae* (83, 317). Strains of *N. meningitidis* with decreased susceptibility to penicillin have been described worldwide. In Canada, the incidence reached 18% in 1995 (24), 43.6% in Spain (90); however in some other countries it is still very low with a prevalence of 4.3% in the Netherlands (303). Both β-lactamases and modified PBPs were identified in this species (219). These facts underline the importance of monitoring the susceptibility of these pathogens to different antimicrobials before they become resistant to most of the antibiotics available.

1.1.2.2.2 Bacterial gastroenteritis

As for VRE, resistance mechanisms in *Salmonella* spp. and *Campylobacter jejuni,* the most frequent cause of bacterial gastroenteritis, are thought to have been acquired in animals and then transmitted into humans (305). Fluoroquinolone is used as the drug of choice in empirical treatment, and the use of this drug in the farming industry has contributed to the selection of resistant campylobacter and salmonella (80, 299). Resistance to this antibiotic has been rising since the late 1990s due to a point mutation which alters the binding site of the antimicrobial agent (166), the incidence has now reached 50% for *Campylobacter* spp. in UK (221). Fluoroquinolone resistance in Salmonella spp. has increased only in the recent years (170). However the situation is worrying in salmonella with the increase worldwide of strains resistant to different classes of antibiotics (104, 298, 335). Erythromycin is the first line antibiotic used to treat infections caused by campylobacter, and resistance to this antibiotic has been observed (47); although the incidence is still low, the increase in resistance narrows the choice of antibiotics for treatment.

The extensive use of antimicrobial agents both in the hospital environment and in the community has led to the emergence of antibiotic-resistant bacterial infections. Emergence of resistance limits the therapeutic options available to physicians in the treatment of infections caused by such pathogens. Therefore, careful monitoring of these pathogens and their susceptibilities as well as appropriate and prudent use of antibiotics is required to constrain and prevent the emergence of resistance.

1.1.2.3 In the agricultural environment

Antimicrobials are also used in food animals to treat or prevent diseases and also to promote growth (132). The most commonly used antimicrobial drugs in animals reared for food are from six major classes: aminoglycosides, β-lactams, tetracyclines, macrolides, sulphonamides and quinolones (132). In 1969, the Swann Committee of the United Kingdom gave a warning about the cross-resistance exerted by the same antibiotics used in animals and humans (287). Although it is difficult to establish a direct link between antibiotic resistance in humans and in animals, as noted above, some studies have shown that antibiotic use in agriculture can act as a driving force for the development of antibiotic resistance in certain pathogenic bacterial species (129, 130, 288, 305, 318).

1.1.2.3.1 In animals

The acquisition of the same mechanism of resistance in animal and human bacteria is evidence of genetic exchange between organisms from these two different environments although exchange via an intermediate organism can also take place. Enterococci are a dominant bacterial group in the intestinal microbiota of humans and animals and VRE have emerged as an important human pathogen due to selection pressure exerted by the use of vancomycin in hospitals (27). However in Europe a glycopeptide, avoparcin, has been used as a growth promoter in animal feed, and its use was shown to create a reservoir of VRE in animals (13). Avoparcin resistance is mediated by the *vanA* gene located on a transposon, Tn*1546* (332). These genetic elements are indistinguishable from the ones found in human isolates

(130). *vanA* was also isolated from sewage (320), meat products (316, 320), and from turkeys and farmers on farms where avoparcin was used as an antimicrobial growth promoter (281).

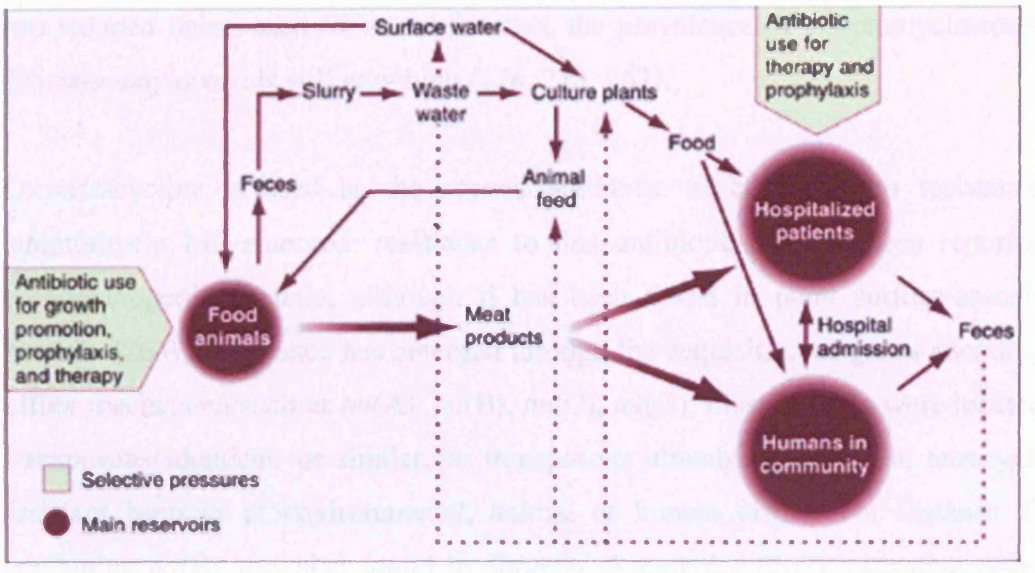
The fact that pulse-field gel electrophoresis (PFGE) of ciprofloxacin-resistant *E. coli* from poultry and poultry farmers and broiler and broiler farmers had identical patterns is evidence that identical clones were present in humans and in poultry (304).

Recently the genomes of MRSA isolates from food animals compared by random amplified polymorphic DNA (RAPD) were found to be closely related to those of human MRSA in Korea (124) and thus might be a possible source of infections to humans via contaminated food products.

The observation of the same clones of *Salmonella newport* serotype MDR-AmpC, which is multidrug resistant, in dairy cattle, in animals and humans (336) further demonstrates that animal pathogens could be a source of infections in humans.

There is good evidence that the use of antimicrobial agents in animals for the treatment or prevention of infections or for growth promotion has encouraged the establishment of a reservoir of antibiotic-resistant bacteria and antibiotic resistance genes in animals that can be transferred to humans directly or indirectly as described in Figure 1.2.

Figure 1.2: Transfer of resistant determinants between different reservoirs



From Witte (329).

As discussed in chapter 1.1.2, the use of antibiotics has created reservoirs of antibiotic-resistant bacteria and antibiotic resistance genes in each environment where antimicrobials are used; this figure underlines the transfer of antibiotic-resistant organisms between these different environments.

1.1.2.3.2 In plants

An estimated 0.1% of the total antimicrobials used in the United States are used in plant agriculture (309). Streptomycin, an aminoglycoside antibiotic, and oxytetracycline, a tetracycline antibiotic, are the main antibiotics used to prevent bacteria-induced rotting of commercially valuable fruits (263). Because of its high efficacy and low toxicity, streptomycin has been the antibiotic of choice; however its repeated use for fire blight control in pears and apples has led to the establishment of streptomycin-resistant *Erwinia amylovora* populations, the causative agent of the fire blight disease. The resistance also spread to other phytopathogenic bacteria and is due to either a mutation in the ribosomal binding site or due the presence of a Tn*3*-type transposon, Tn*5393*, containing linked *strA-strB* genes that encode streptomycin-inactivating phosphotransferases (176). Tn*5393* is located on a plasmid, pEa8.7 that is closely related or identical to the broad-host range plasmid

RFS1010, which is commonly found in a variety of clinically important bacteria of human and animal origin (44, 213). The concerns are that even though streptomycin has stopped being used for some decades, the prevalence of streptomycin-resistant *Erwinia amylovora* is still very high (176, 213, 263).

Oxytetracycline is used as the second antibiotic of choice when resistance to streptomycin has emerged; resistance to this antibiotic has not been reported in phytopathogenic bacteria, although it has been found in plant surface-associated bacteria (263). Resistance has emerged through the acquisition of genes encoding an efflux mechanism such as *tet*(A), *tet*(B), *tet*(C), *tet*(G); most of them were located on transposons identical, or similar, to transposons already described in tetracycline-resistant bacteria of environmental, animal or human origin. For instance Tn*10* containing *tet*(B) was also found in *Shigella dysenteriae* (263), causative agent of gastroenteritis in humans and Tn*1720*, a variant of Tn*1721*, containing *tet*(A) was also found in *Salmonella enterica,* also causative agent of gastroenteritis in humans (220). In some cases, tetracycline resistance was linked to streptomycin resistance determinants and so the use of oxytetracycline in plant agriculture would select for tetracycline and streptomycin resistance (263).

Many different factors have contributed to the emergence of antibiotic-resistant bacteria as seen above. The presence of homologous resistance determinants in bacteria associated with plants, soil, animals and humans is likely to be due to gene exchange between organisms from these different environments, therefore different habitats can serve as a reservoir for antibiotic resistance genes, which can then be transmitted to other environments (Fig. 1.2).

1.2 Origins of the rise of antibiotic-resistant bacteria

1.2.1 Point of view of the bacterium

Bacteria are part of us; human beings are made up of 10^{14} cells, only 10% of these are mammalian cells, the remainder are the microbes which constitute the indigenous

microbiota (155). Bacteria have a short generation time and so are able to adapt efficiently to a change in the environment, which means they can become more or less resistant in response to selection pressures exerted by the presence or absence of an antibiotic in their environment.

1.2.1.1 Bacteria challenged by antimicrobial agents

1.2.1.1.1 Increase in the proportion of antibiotic-resistant bacteria

When an antibiotic is administered, the entire microbiota is exposed to the compound; it first affects the susceptible bacteria, by either preventing them from growing any further or by killing them, thus giving additional resources and more opportunity for the resistant bacteria to proliferate in this new environment (155). The administration of tetracycline to an oral biofilm *in vitro* was shown to increase the overall resistant population and decrease the susceptible one (230). The second effect caused by the consumption of an antibiotic is an increase in antibiotic resistance gene exchange between bacteria. One study on the effect of tetracycline on *Bacteroides* spp. showed that exposure of the bacteria to a low, subinhibitory concentration of the drug upregulates the expression of the gene transfer apparatus of the conjugative transposon CTnDOT (280, 323); also tetracycline was shown to increase the transfer frequency of Tn*916* in a culture of *Bacillus subtilis* (39). Thus antimicrobial agents create a selective pressure on the bacterial population favouring the growth of resistant bacteria and encouraging the bacteria to acquire new mechanisms of resistance.

1.2.1.1.2 Origin of the antibiotic resistance genes

Plasmids isolated during the pre-antibiotic era were common but carried very few antibiotic resistance genes; out of 433 enterobacterial strains studied, including those belonging to the genera *Proteus*, *Salmonella*, *Escherichia*, *Klebsiella*, from the period of 1917-1952, only one *Proteus* strain was shown to be resistant to tetracycline, one *Klebsiella* strain was resistant to ampicillin and no other antibiotic

resistance was found (123). Recently the urinary pathogens *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* were studied (864, 71 and 62 isolates respectively) in the UK for their resistance to different antimicrobials and it was found that 25.6% of *E. coli*, 18.3% of *K. pneumoniae* and 100% of *P. mirabilis* were resistant to tetracycline and resistance to amoxycillin, ampicillin and trimethoprim were found in all species (82), suggesting that they have acquired resistance genes following the increased use of antibiotics. The origin of these resistance genes is not well understood. There is some evidence that most of them originated from the antibiotic-producing organisms as a way to protect themselves against the activity of the antibiotics they produce (64). The best example is the striking similarity of the genes conferring vancomycin resistance (*vanA*, *vanB*, *vanH, vanX*) in enterococci and the related clusters in glycopeptide antibiotic producers (171, 172). In some cases it has been shown that antibiotic resistance genes might have evolved from housekeeping genes (64). The aminoglycoside modifying enzymes, aminoglycoside acetyltransferases and aminoglycoside phosphotransferases, have significant primary sequence similarities to the housekeeping genes acetyltransferases and kinases respectively found in streptomycetes (63), now these two antibiotic resistance genes can be found in a wide variety of species (248).

1.2.1.1.3 Cross-resistance

One major problem is the co-selection for multiply-resistant organisms (25, 248). The antimicrobial agents have selected for the assembly and evolution of complex genetic vectors encoding, expressing, linking and spreading different resistance genes. It is not uncommon to isolate one bacterium resistant to more than one antibiotic eg MRSA, VRE, multidrug-resistant Salmonella, penicillin-resistant *Streptococcus pneumoniae* (325) and it is easy to understand how, considering the array of genetic elements able to carry and transfer antibiotic resistance genes and the presence of gene cassettes able to accumulate different resistance genes and to subsequently transfer them to transposons and plasmids (see chapter 1.2.2.2). The linkage of antibiotic resistance genes on the same element is of concern; the use of any of the antibiotics can select for multidrug-resistant isolates and aid the survival of resistance genes even in the absence of the drug to which they encode resistance.

Moreover, biocides and antibiotics use some common mechanisms of resistance such as the QAC (quaternary ammonium compounds) efflux pumps (Table 1.4) and some genes coding for resistance to biocides or disinfectants have also been linked with antibiotic resistance genes; the quaternary ammonium compounds resistance gene (*qacA/B*) was linked to β-lactam resistance genes on the same plasmid in food-related *Staphylococcus* spp. (270) therefore co-selection for multidrug-resistant strains is also likely to take place in any environment where biocides are used (153, 177, 222). The use of mercury in dental amalgams is likely to promote the development and maintenance of mercury- and antibiotic-resistant bacteria in the oral cavity; there are some reports of antibiotic-resistant bacteria also being resistant to mercury (283) and mercury resistance has been associated with conjugative plasmids or/and transposons in the environment (212).

1.2.1.2 Mechanisms of resistance

There are various ways for bacteria to protect themselves from the effect of an antibiotic: the production of enzymes that inhibit the activity of the agent, the active efflux of the drug from the cell, a decreased uptake of the drug, the creation of an alternative metabolic pathway that bypasses the target action or the modification of the target site preventing the antibiotic from binding to it.

1.2.1.2.1 Intrinsic and acquired resistance

Acquired resistance is present only in certain isolates of a species or genus.

A bacterium can be resistant to an antibiotic through an intrinsic or acquired mechanism. Intrinsic resistance is present in all the members of a given species or genus. For example enterococci are intrinsically resistant to different antibiotics such as penicillin because the β-lactam antibiotics bind very weakly to enterococcal penicillin-binding proteins involved in bacterial cell wall synthesis, to macrolides because of a modified ribosomal target and finally to aminoglycosides which cannot efficiently cross the cell wall (69). Intrinsic resistance differs in Gram-negative and

Gram-positive bacteria because of their differing cell wall composition (225). In both Gram-positive and Gram-negative organisms, the envelope consists of a peptidoglycan layer, a periplasmic space followed by the inner plasma membrane, however in Gram-negative bacteria the peptidoglycan layer is surrounded by an extra outer membrane that consists of lipids, lipoproteins, lipopolysaccharides and porin proteins. One important function of the outer membrane is to prevent or slow the entry of bile salts, antibiotics and other toxic substances. Aminoglycosides and glycopeptides are high hydrophilic compounds which prevents them from crossing the highly lipophilicity of the outer membrane of most Gram-negative organisms therefore they are inactive against most of these organisms (63, 83).

1.2.1.2.2 Active efflux of the drug

Efflux pumps, or energy-dependent export, evolved to protect bacteria from the toxic substances resulting from their own metabolism (156). However, some of these pumps excrete antimicrobial agents out of the cells fortuitously, thus preventing the intracellular accumulation necessary for antibiotics to exert their lethal activity and conferring diminished susceptibility to these different compounds.

The efflux pumps can be divided into four superfamilies according to their amino acid sequence morphologies (Table 1.4). Most of these efflux pumps (MFS major facilitator superfamily, SMR small multidrug resistance protein family and RND resistance, nodulation, division) are proton motive force-dependent efflux systems; that is, systems that use the chemical gradient of hydrogen ions and/or the electrical charge gradient across the membrane to drive drug efflux (150, 229). Whereas, ABC transporters (ATP-binding cassette), which contain two ATP binding cassettes, derive their energy from the hydrolysis of ATP (Fig 1.3). Although the mechanisms of antibiotic substrate specificity are difficult to establish, it is thought that transporters recognise molecules with a polar, often slightly charged head, associated with a hydrophobic domain (156, 217).

Efflux pumps can have an important role in intrinsic resistance of Gram-negative bacteria to different antibiotics. Among Gram-negative bacteria, three types of

multidrug efflux pumps have been reported (Table 1.4): MFS, SMR and RND, which work in synergy with the membrane barrier, giving rise to increased resistance to a wide range of antibiotics. Efflux systems of Gram-positive organisms belong either to the MFS, SMR or ABC families (Table 1.4); they usually exhibit narrow substrate specificities and confer resistance to weakly lipophilic agents or organic cations. Elevated expression of some of these efflux proteins can be caused by a mutation in the promoter region as is the case of the NorA protein (133) or by a mutation in the regulator of the genes encoding the efflux pump, as is the case for the Mex pump (338). The broad-substrate range of efflux systems is of concern, since overexpression of a pump will result in resistance to antibiotics of more than one class as well as to some dyes, detergents and disinfectants (Table 1.4). Moreover, it underlines the risk of commonly used biocides that could also select for cross-resistant bacteria (92, 153).

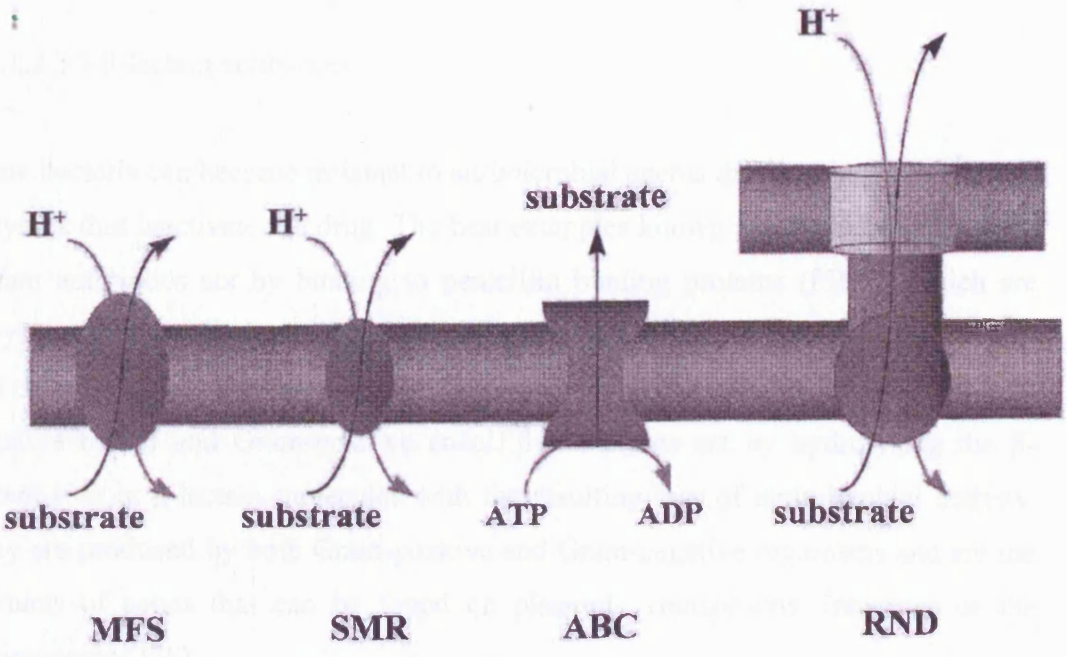
Table 1.4: Multi-drug resistance families and members

MDR families	Properties	Membrane topology	Distribution	Determinant	Resistance Phenotype	Microorganims
MFS	• PMF • And specific translocases	12-14 transmembrane helices	prokaryotes and eukaryotes	*qac*(A)/*qac*(B) *nor*(A) *pmr*(A) *bmr*, *blt* *mef* *mdr*(l) *tet*(K)/tet(L) *tet*(A)-*tet*(E)	QAC Quinolone Quinolone Quinolone Macrolide MDR Tet Tet	*Staph.* aureus *Staph. aureus* *Strep pneumoniae* *B. subtilis* *Strep, Neisseriae* *L.monocytogenes* Gram-positive Gram-negative
SMR	PMF	4 transmembrane helices (most likely organized in trimers)	Gram-positive and Gram-negative species	*smr* *qac*(E) *emr*(E)	MDR QAC MDR	*Staph. aureus* Broad host range *E. coli*
RND	PMF	12 transmembrane helices	Gram-negative species	*acr* *mex* *mtr*	MDR MDR MDR	*E. coli* *P. aeruginosa* *N. gonorrhoeae*
ABC	Specific translocases	12 transmembrane helices	prokaryotes and eukaryotes	*msr*(A) *vg*(A)/*vg*(B)	Macrolide Streptogramin A	*Staph. aureus* *Staph* spp.

Adapted from Köhler *et al.* (141), Lewis *et al.* (157), Paulsen *et al.* (217) and VanBambeke *et al.* (302).

MFS = major facilitator superfamily, SMR = small multidrug resistance protein family, RND = resistance, nodulation, division, ABC = ATP-binding cassette, MDR = multidrug resistance, PMF = proton motive force, QAC = quaternary ammonium compounds used as antiseptics, Tet = tetracycline, *Strep* = *Streptococcus* spp., *Staph* = *Staphylococcus*.

Figure 1.3: Structure of bacterial efflux systems



From Köhler *et al.* (141).

The bacterial efflux systems are composed of a cytoplasmic pump protein that allows the export of substrates from within the cell into the external medium. MFS, SMR and RND derive their energy from the proton gradient force whereas ABC uses the hydrolysis of ATP. RND is composed of two additional proteins believed to form a channel as represented in this figure.

Drug efflux appears to be a widespread mechanism of resistance in Gram-positive and Gram-negative bacteria including medically-important species such as staphylococci, streptococci, enterobacteria and opportunistic pathogens like *Pseudomonas aeruginosa* (Table 1.4). Its ability to export unrelated compounds, and to increase bacterial resistance by overexpression of the efflux systems, makes it an important mechanism of resistance to antimicrobial agents and biocides.

1.2.1.2.3 Resistance to classes of antibiotics

1.2.1.2.3.1 β-lactam antibiotics

Some bacteria can become resistant to antimicrobial agents through the production of enzymes that inactivate the drug. The best examples known are the β-lactamases. β-lactam antibiotics act by binding to penicillin binding proteins (PBPs), which are enzymes involved in the terminal stages of assembling the cell wall during growth and division (178); they are bactericidal antibiotics and usually used against Gram-negative bacilli and Gram-positive cocci. β-lactamases act by hydrolysing the β-lactam ring in β-lactam molecules with the resulting loss of antimicrobial activity. They are produced by both Gram-positive and Gram-negative organisms and are the products of genes that can be found on plasmids, transposons, integrons or the chromosomes (28).

The second most common mechanism of resistance against β-lactam antibiotics after the production of inactivating enzymes is the change by mutations, or replacements by transformation, of parts of the various PBP genes leading to a decreased affinity for the antibiotic (28).

1.2.1.2.3.2 Aminoglycosides

This class of antibiotics inhibits protein synthesis in Gram-positive and Gram-negative organisms. The mechanism of resistance to aminoglycosides is through the production of enzymes which modify the antibiotic and usually lead to high-level resistance. These enzymes are classified as adenyltransferases, acetyltransferases and phosphotransferases; they add an adenine, acetyl or phosphate group respectively, onto the aminoglycoside molecule, preventing them from binding to the ribosome target site (63). Within each group there are increasing numbers of different enzymes, which differ in the aminoglycosides that they can use as substrate and the position on the aminoglycoside where the modifying molecule is added. The genes for the enzymes may be located in the chromosome, plasmids, transposons or integrons (63).

Aminoglycoside resistance can also occur by base modification in the ribosomal RNA (rRNA), the binding site of the antibiotic, by an RNA methylase and confer high level of resistance. However, this has not yet been identified in clinical isolates (63).

1.2.1.2.3.3 Macrolides

The macrolides are bacteriostatic; they bind to the bacterial ribosome and consequently inhibit protein synthesis. Erythromycin, one of the macrolides, is effective against Gram-positive cocci and is often used as a substitute for penicillin against streptococcal and pneumococcal infections (138).

The most common mechanism of resistance to macrolides (M) is target modification that leads to the alteration of a site in 23S rRNA common to the binding of macrolides as well as the unrelated lincosamide (L) and streptogramin B (S) antibiotics and thus conferring cross-resistance to MLS antibiotics (247). This type of resistance is conferred by the presence of rRNA methylases (encoded by *erm* genes) that modify a single adenine residue at position 2058 in the 23S rRNA and prevent MLS antibiotics from binding to the 50S ribosomal subunit. There are around 20 different *erm* genes coding for rRNA methylases; they have been isolated from a variety of bacteria and are associated with conjugative or nonconjugative transposons (Table 1.5) that tend to reside on the chromosome although some have been found on plasmids (247).

Inactivating enzymes produced against macrolides are very rare although some have been found in lactobacilli, staphylococci and *E. coli* (11, 247, 330). They are plasmid-mediated erythromycin esterases typeI and typeII (*ereA* and *ereB* respectively) (11) or transposon-mediated macrolides 2'-phosphotransferase (*mphA* and *mphB*) (204, 205) and they confer high levels of resistance to 14-membered macrolides which are erythromycin and clarithromycin (*ereA, ereB, mphA, mphB*) and to 16-membered macrolides which are spiramycin, josamycin and tylosin (*mphA, mphB*) (Table 1.5).

A third mechanism of resistance to macrolides is through the production of efflux proteins that pump the antibiotics out of the cell of which there are two types. One set of proteins, coded by the *mef* gene, has homology with the major facilitator superfamily; they are found in *Streptococcus* spp. and oral *Neisseria* spp. and are associated with conjugative elements found in the chromosome (247). The second set of proteins, coded by the *msr* gene, belongs to the ABC transporter family, but has been isolated so far only from *Staphylococcus* spp. (Table 1.4).

A fourth mechanism of resistance, a base substitution in the 23S rRNA where macrolides make several contacts with the ribosome, has emerged in clinical isolates since the 1990s (247). The most common mutation is at position A2058; methylation of the rRNA at A2058 by *erm* methyltransferases is thought to confer resistance by a similar mechanism. Mutation conferring resistance to macrolides can also appear in the ribosomal proteins (L4, L22) (308, 319). These mutations lead to different phenotypes (36, 308).

Intrinsic resistance to MLS antibiotics in Gram-negative bacilli is due to low permeability of the outer membrane to these hydrophobic compounds (148).

Table 1.5: Distribution of erythromycin resistance genes

Class of macrolide resistance gene[a]	Protein	Gene name	Localisation	Species where the gene resides	Reference
Methylases	ErmA	*erm*(A)[b]	Tn*554*	*Actinobacter, Staph, Strep, Peptostrep*	247, 233
	ErmB	*erm*(B)	Tn*1545*, plasmid	Broad host-range	247
	ErmC	*erm*(C)	Plasmid	Broad host-range	247
	ErmF	*erm*(F)	Tn*4551*, plasmid, chr, Tn	Broad host-range	48, 324
	ErmQ	*erm*(Q)	chr	*Actinomyces, Strep*	247
	ErmT	*erm*(T)	Plasmid	*Lactobacillus*	247
Inactivating Enzymes	EreA	*ere*(A)	pIP1100	*Enterobacteriacae*	247
	EreB	*ere*(B)	pIP1527	*Enterobacteriacae*	247
	MphA	*mph*(A)	Tn	*E. coli*	205
	MphB	*mph*(B)	Plamid, Tn	*E. coli*	203, 204
	MphC	*mph*(C)	ND	*Staph*	247
Efflux pumps	Mef[c]	*mef*(A)	Tn*1207.3* Tn*2009*	*Enterococcus, Strep, Staph, Neiss, Acineto*	251 68
	MsrA	*msr*(A)	pSR1, pEP2104	*Staph*	247

[a] The distinction of the *erm* methylases is based on their amino acid sequence variability (247)

- two genes with <80% amino acid sequence identity provide enough variability to permit distinct probes to be designed
- if two genes have an amino acid sequence identity >=80%, they are assigned to the same class and same letter designation
- if two genes have an amino acid sequence identity <=79%, they are given a different letter designation.

[b] *erm*(TR) is also designated *erm*(A) according to current nomenclature (247).

[c] *mef*(A) originally described in *S. pyogenes* (49) and *mef*(E), originally described in *S. pneumoniae* (247), are 90% identical and were assigned the same *mef*(A) class of macrolides resistance determinants (247).

Chr = chromosome, Tn = Transposons, *Staph* = *Staphylococcus, Strep* = *Streptococcus, Neiss* = *Neisseria, Acineto* = *Acinetobacter, Peptostrep* = *Peptostreptococcus*, ND = not determined.

1.2.1.2.3.4 Tetracyclines

Tetracyclines are bacteriostatic, inhibiting bacterial protein synthesis. They are broad-spectrum antibiotics effective against a wide range of Gram-positive and Gram-negative bacteria (45). The production of ribosomal proteins that bind and protect the ribosomes from the action of tetracycline is very common as a mechanism of resistance to this antibiotic in both Gram-positive and Gram-negative bacteria (274). These ribosomal proteins have extensive homology to the elongation factor G-like proteins that are required for the correct binding of aminoacyl-tRNA to the ribosomal acceptor site in the presence of guanosine triphosphate (GTP) (45, 139). So far, nine classes of genes encoding ribosome protection proteins (RPP) have been described: *tet*(M), *tet*(O), *tet*B(P), *tet*(Q), *tet*(S), *tet*(W), *tet*(T), *tet*(32) and *tet*(36) (Table 1.6). The most common of which is *tet*(M) (45, 249). One of the reasons for the success of these genes is the fact that they are commonly contained within conjugative transposons, which have an extraordinarily broad host range (45, 236, 274).

Enzymatic inactivation of tetracycline is encoded by two determinants similar in their functions: Tet X and Tet 37. The *tet*(X) gene was isolated from the anaerobic intestinal bacteroides transposons (275) whereas *tet*(37) was cloned from the oral metagenome (72). Both gene products chemically modify tetracycline in the presence of both oxygen and nicotinamide adenine dinucleotide phosphate (NADPH). However, to date no surveys have been conducted to assess the distribution of these two genes.

A third mechanism of resistance to tetracycline is through the production of efflux proteins (Table 1.6). They are encoded by different tetracycline resistance genes, *tet*(A) to *tet*(I), including *tet*(39) found mostly in Gram-negative species and *tet*(K) and *tet*(L) widely distributed among Gram-positive organisms. They all belong to the same family of the MFS (Table 1.4). The Gram-negative efflux genes are normally associated with large plasmids, most of which are conjugative, while the Gram-positive efflux genes are generally found on smaller transmissible plasmids (45).

A novel *tet* gene *tet*(34) has recently been described from a *Vibrio* sp. (206); the possible function of this determinant is activation of Mg^{2+}-dependent purine nucleotide synthesis, thus providing an excess of guanosine triphosphate stimulating the elongation factor proteins required for the binding of aminoacyl-tRNA to the ribosomal acceptor site. Consequently, an excess supply of GTP might accelerate the binding of aminoacyl-tRNA and elongation factor, which would attenuate the inhibition by tetracycline antibiotics (206).

Finally, tetracycline resistance can also arise through mutation at the ribosomal binding site of the antibiotic. These mutations have been found in two different clinical species, *Propionobacterium acnes* (252) and *Helicobacter pylori* (99), and were shown to have a different effect on bacterial fitness, depending on the type of mutation carried (99).

Table 1.6: Distribution of tetracycline resistance genes

Class of tet resistance gene	Protein	Gene	Localisation	Species where the gene resides	Ref
Ribosomal protective proteins [a]	Tet M	*tet*(M)	Plasmid, CTn	Broad host-range	45
	Tet O	*tet*(O)	Plasmid, CTn	Broad host-range	249
	Tet P	*tetB*(P)	Plasmid	*Clostridium*	154
	Tet Q	*tet*(Q)	CTn	Broad host-range	151
	Tet S	*tet*(S)	Plasmid, CTn, chr	*Listeria, Enterococcus, Streptococcus*	43, 144
	Tet T	*tet*(T)	ND	*Streptococcus*	45
	Tet W	*tet*(W)	CTn, associated with mob proteins	Rumen bacteria, human faeces bacteria, animal pathogen	17, 21, 264
	Tet 32	*tet*(32)	ND	*Clostridium*	180
	Tet 36	*tet*(36)	ND	*Cytophaga- Flavobacter-Bacteroides, Proteobacteria*	322
Inactivating enzymes	Tet X	*tet*(X)	Tn	*Bacteroides*	275
	Tet 37	*tet*(37)	ND	ND	72
Efflux pumps [a]	Tet A	*tet*(A)	Plasmid	Gram-negative	45
	Tet B	*tet*(B)	Plasmid, Tn, chr	Gram-negative	45
	Tet C	*tet*(C)	Plasmid	Gram-negative	45
	Tet D	*tet*(D)	Plasmid	Gram-negative	45
	Tet E	*tet*(E)	Plasmid, chr	Gram-negative	45
	Tet F	*tet*(F)	Plasmid	*Bacteroides*	45
	Tet G	*tet*(G)	Plasmid	*Vibrio, Pasteurella*	45, 136
	Tet H	*tet*(H)	Plasmid, Tn	*Pasteurella*	136
	Tet I	*tet*(I)	Plasmid	*Providencia*	249
	Tet J	*tet*(J)	Unsequenced	*Proteus*	45
	Tet K	*tet*(K)	ND	Gram-positive	45
	Tet L	*tet*(L)	Plasmid	Gram-positive	45
	Tet P	*tetA*(P)	Plasmid, chr	*Clostridium*	154
	Tet V	*tet*(V)	Plasmid	*Mycobacterium*	154
	Tet Y	*tet*(Y)	ND	*E. coli*	154
	Tet Z	*tet*(Z)	Plasmid	*Corynebacterium*	154
	Tet 30	*tet*(30)	Plasmid	*Agrobacterium*	154
	Tet 33	*tet*(33)	Tn	*Corynebacterium*	291
	Tet 39	*tet*(39)	Plasmid	*Acinetobacter*	4
Mg^{2+} dependent resistance	Tet 34	*tet*(34)	ND	*Vibrio*	206
Unknown mechanism	Tet U [b]	*tet*(U)	Plasmid	*Enterococcus*	238

[a] The distinction of the *tet* genes is based on their amino acid identity and <= 80% is chosen as the dividing line (154).

[b] The resistance mechanism of this gene appears to be ribosomal protection however the sequence of the gene shows no homology to the elongation factor G-like proteins (238).

tet = tetracycline, Ref = References, Chr = chromosome, Tn = Transposons, CTn = Conjugative Transposons, ND = not determined.

1.2.1.2.3.5 Glycopeptides

Glycopeptides interfere with the synthesis of the bacterial cell wall by binding to the D-alanines on the precursors of the peptidoglycan cross bridges preventing their cross-linking and leading to cell lysis (235). Vancomycin was introduced clinically in 1958 for the treatment of infections due to Gram-positive bacteria (277). Use of this agent has increased dramatically in the last 20 years, in large part because it has become the antibiotic of last resort for infections due to some organisms (e.g. MRSA) as resistance to the other antibiotics has become more common. The only known acquired mechanism of resistance to glycopeptides in Gram-positive organisms is through the modification of the drug target (91). Resistance has developed through the acquisition of genes (*vanA*, *vanB*, *vanD*, *vanE*) that produce a ligase resulting in the modification of the peptidoglycan side chain with less affinity to glycopeptides (91). These genes confer different levels of resistance and were described in different microorganisms (100, 223, 295). The most common of those is *vanA* which is encoded by Tn*1546*, resulting in high level of resistance in *Enterococcus* spp. (69).

Gram-negative organisms are intrinsically resistant to vancomycin since the peptidoglgycan inside the outer cell membrane cannot be reached by the glycopeptide molecule (91). Natural resistance exists in Gram-positive organisms such as *Lactobacillus* spp. due to a lower affinity to glycopeptide antibiotics (22).

1.2.2 Mechanisms of disseminating antibiotic resistance genes

1.2.2.1 Discovery of bacterial gene exchange

Until the discovery of transformation in the early 1940's, it was assumed that bacteria lacked any means of exchanging genetic material. Within a few years, two additional means of genetic exchange by bacteria, conjugation and transduction, were discovered. All three of these mechanisms result in the transfer of DNA from a donor to a recipient bacterium (240). The extent of horizontal gene transfer (HGT) was further demonstrated by the characterisation of nucleic acids in bacterial genomes (146). Sequencing and comparisons of genomes from different species revealed regions with atypical base compositions or codon usage patterns, which have been described as 'alien genes' and is evidence of a HGT between bacteria (146). Bacterial genomes are extremely dynamic and mosaic in nature, HGT are often involved in the rapid adaptation of bacteria to novel environments and thus are likely to play a major role in the acquisition of antibiotic resistance.

1.2.2.2 Mechanisms of HGT

1.2.2.2.1 Transformation

Transformation is a process by which bacteria can take up naked DNA from the environment. Many bacteria are naturally transformable; they can take up DNA during a specialised physiological state termed competence, the development of competence depends on different factors according to the species (272). More than 40 naturally transformable species have been identified (163).

Different steps are involved for successful transformation to take place. First of all, free bacterial DNA in the environment is required and it is usually provided by the lysis of bacterial cells. The DNA must persist in the environment and be protected from the action of free nucleases. Some studies showed that adsorbed DNA on surfaces such as minerals, sand and clay particles is less susceptible to the action of DNAse, however the mechanisms of protection are unknown (251).

Extracellular DNA can be taken up efficiently only by competent bacteria; in most bacteria competence is inducible, but in *N. gonorrhoeae*, and probably *Acinetobacter* spp., it is constitutive (272). In transiently competent bacteria, competence develops when the cell is in the correct physiological condition (76), but it also depends on the expression of genes whose proteins provide the necessary functions to induce competence (50, 272). These proteins are produced from the *com* genes in *B. subtilis*, and they also have analogues in other species (77, 78).

In *B. subtilis* and *S. pneumoniae*, the binding of DNA onto the cell surface is non covalent, it takes place through the presence of binding proteins encoded by the *com* genes (226) whereas the interaction between DNA and a competent cell in *N. gonorrhoeae* and *H. influenzae* occurs through the presence of short specific DNA-uptake sequences (DUS) in the donor DNA which is why *N. gonorrhoeae* and *H. influenzae* preferentially take up double-stranded DNA only from the same or closely related species (20, 272).

In most species, after binding to the cell surface of competent cells, the DNA undergoes double-strand cleavage. While one strand of the bound DNA is degraded to acid soluble products, the other strand becomes resistant to DNAse and is transported across the cytoplasmic membrane (163). The uptake is dependent on monovalent and divalent cations in most species and is similar in Gram-positive and Gram-negative organisms. One marked difference in the uptake system of *H. influenzae* and *N. gonorrhoeae* is that the donor DNA is taken up as a double strand into vesicles called transformasomes which are located onto the cell surface. Thus the DNA contained in the transformasome is protected against exogenous DNAse. However, once in the cytoplasm the DNA becomes single stranded (163).

Integration of the donor DNA into the recipient chromosome is via a homologous recombination that is dependent on the activity of the RecA protein: single stranded molecules interact with complementary sequences in the recipient chromosome to yield heteroduplex DNA, while the old strand with the same sequence is displaced and subsequently degraded. This recombination appears to be identical in Gram-positive and Gram-negative bacteria (163). Also bacteria can be transformed by

plasmids, which then replicate and do not need homologous recombination to become established (163).

HGT by transformation has been demonstrated in different bacteria in a variety of natural ecosystems (Table 1.7) and also in the laboratory among different bacteria (163), showing that it is an important factor in the evolution of bacteria, including the acquisition of antibiotic resistance.

Table 1.7: Transformation in natural environments

Bacterial host	Environmental Situation	Genetic marker	Reference
P. stutzeri	Marine water microcosm	Chromosomal RifR	65
Pseudomonas sp.	Marine Water and sediment microcosm	Plasmid multimers	65
A. calcoaceticus	Ground water and soil extract	Chromosomal *trp*	65
A. calcoaceticus	Ground and aquifer water	Plasmid	65
A. calcoaceticus	River epilithon	Chromosomal *his*	65
A. calcoaceticus	Soil microcosm	Chromosomal DNA + KmR, GmR cassette	65
E. coli	River and spring water	Plasmid	65
P. stutzeri	Soil microcosm	Chromosmal his and plasmid	65
A. tumefaciens *P. fluorescens*	Soil microcosm	Plasmid	70
Acinetobacter sp.	Soil microcosm	Chromosomal + KmR cassette	198
S. gordonii	Human saliva	Plasmid	181
Acinetobacter sp.	Soil and rhizospheres	Plasmid KmR	71

Table adapted from Davison *et al.* (65).

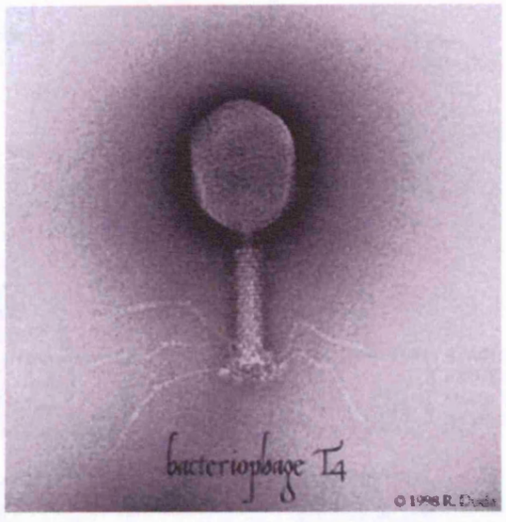
RifR = rifampicin resistance, *trp* = tryptophan, *his* = histidine, KmR = kanamycin resistance, GmR = gentamicin resistance.

1.2.2.2.2 Transduction

Transduction involves the transfer of DNA from one bacterium by a bacteriophage (a virus that infects bacteria). There is a wide variety of bacteriophages (250), some contain single- or double-stranded RNA or DNA, but only double-stranded DNA (dsDNA) containing phages can mediate transduction and thus will be the focus of this section. Tailed phages are the most efficient transducing vectors (115). Their structure reveals a densely compacted phage DNA, encased in a protective protein shell protecting the DNA from degrading enzymes. The phage tail and its associated fibres (Fig 1.4) assure both the specific recognition of the appropriate host cell and the guided injection of the phage DNA into the bacterial cell (152).

Phages can be divided into two general classes, the lytic (or virulent) and temperate (or lysogenic) (34). In both cases there must be interaction between specific phage proteins and receptors on the host cell. This interaction triggers a process that allows infection of the viral DNA or dsDNA into the cytoplasm of the host bacterium. The dsDNA enters the target cell as a linear form, however the ends of the DNA are single stranded and cohesive (they are complementary to one another) therefore the phage DNA is able to circularise by means of the cohesive ends (258). In the case of a lytic phage, the phage DNA directs the biosynthesis of viral parts using the host cell's machinery (protein coat, chromosome); the phages mature as the parts are assembled causing the cell to lyse and release approximately 10^2 to 10^3 progeny (Fig 1.5). In lysogeny, the phage DNA remains latent in the host until it breaks out in a lytic cycle; the lysogenic phages possess the same ability as the lytic phages, however they have the ability to integrate into the host chromosome, usually by a site-specific recombination event which results in an integrated phage termed the prophage. When the prophage becomes activated it excises from the chromosome and enters the lytic cycle (Fig 1.5). Some prophages are maintained as plasmids within the host cell (103).

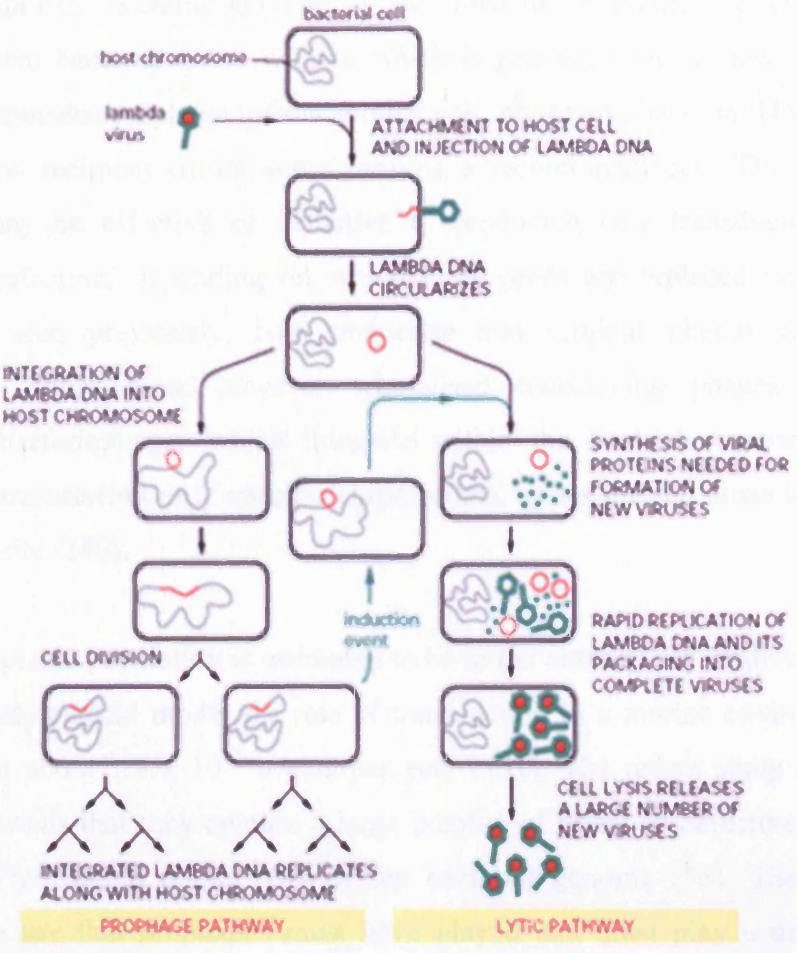
Figure 1.4: Electron micrographs showing the structure of dsDNA phages (T4 phage at the top and λ phage at the bottom)



From http://www.asm.org/division/m/foto/T4Mic.html

From http://www.asm.org/division/m/foto/UrLamMic.html

Figure 1.5: The life cycle of dsDNA phages



Taken from http://www.accessexcellence.org/RC/VL/GG/bact_Lambda.html

This diagram represents the two different life cycles of phage λ

How could transduction be involved in the evolution of bacteria? During the lytic process the phage DNA encodes a nuclease, which causes fragmentation of the bacterial DNA, therefore the host's DNA may be packaged in place of the phage's. If this transducing phage attaches to a susceptible bacterial cell, the exogenous bacterial DNA may be inserted into the susceptible bacterium and be integrated into its chromosome. This process is called generalised transduction and because the phage does not contain a complete phage genome, the transducing particles are defective, no progeny phages are produced and no cell lysis takes place, therefore a horizontal gene transfer event has occurred (240).

In the second type of transduction, specialised transduction, a lysogenic phage is integrated into the bacterial genome so that when the prophage excises it can take some adjacent bacterial DNA with it, which is packaged into a new phage head. When this transducing phage infects another cell, phage and bacterial DNA integrate into the new recipient chromosome forming a recombinant cell. The transducing particles may be effective or defective in producing new transducing particles following infection, depending on which viral genes are replaced with bacterial DNA. As seen previously, both temperate and virulent phages can perform generalised transduction; however specialised transducing phages are solely temperate bacteriophages which integrate within the host chromosome and are capable of transferring only specific chromosomal genes located close to the phage integration site (240).

The global phage population is estimated to be in the order of 10^{31} individuals (115). Using a mathematical model the rate of transduction in a marine environment was estimated at about 1.3×10^{14} events per year (131). The recent study of bacterial genomes reveals that they contain a large number of intact or defective prophages; phages can constitute up to 16% of the bacterial genome (35). Therefore it is tempting to say that prophages must have played and must play a major role in bacterial evolution (35, 37) including the acquisition of antibiotic resistance genes, although examples are scarce (see chapter 1.3.2.1.3).

1.2.2.2.3 Conjugation

Bacterial conjugation is defined as the direct transfer of DNA from the cytoplasm of a donor bacterium to the cytoplasm of a recipient cell and it is mediated by specialised genetic elements; it is thought to be the primary route of broad host range DNA transfer between different genera of bacteria (65). This process is normally specified by plasmids and conjugative transposons.

1.2.2.2.3.1 Plasmids

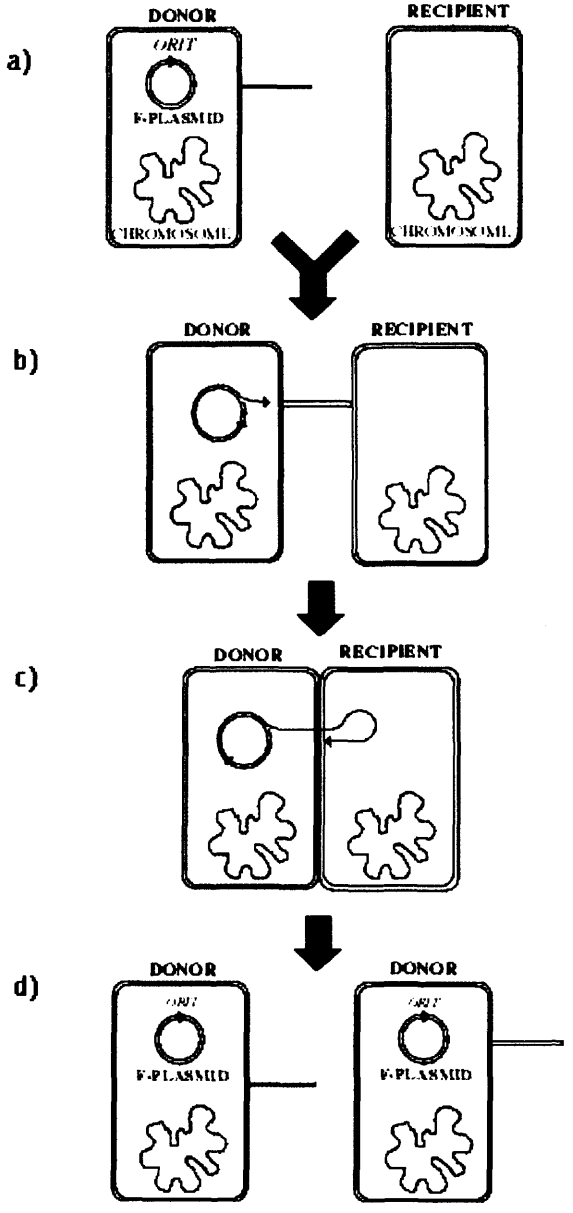
Plasmids are circular dsDNAs that replicate independently from the host chromosome and carry genes that sometimes benefit the host. They have their own origin of replication (*oriV*) and can range in number from one to many per cell (108). Some plasmids have the ability to move from one cell to another by conjugation. Conjugation involves the transfer of the plasmid as single-stranded DNA. A well-studied conjugative plasmid is F from *E. coli*; as well as *oriT*, it also contains a series of *tra* genes, which are essential for plasmid transfer (183), some of which encode a sex pilus that makes contact with a potential recipient cell that does not have an F plasmid. The pilus then contracts and pulls the cells together so that DNA transfer can take place (Fig 1.6). The particularity of the pilus system is its close resemblance to the type IV secretion system (108); this type of secretion family includes conjugation machines and ancestrally related systems that deliver effector molecules to eukaryotic cells (46).

The transfer of plasmid DNA starts at the *ori*T. The *tra* genes do not only encode the proteins necessary for the synthesis and assembly of the sex pili, but they are also involved in the relaxosome formation and, subsequently, DNA replication (87). The relaxosome is the pre-transfer complex at the *ori*T; there is cleavage at the *ori*T by a 'nicking' enzyme, accessory proteins bind to the *ori*T region allowing DNA transfer to start, a DNA helicase unwinds the dsDNA and liberates the ssDNA that is subsequently transfered to the recipient cell (87). Both strands are replicated, resulting in a copy of the plasmid in both the donor and the recipient cell (Fig 1.6). Conjugative transfer between Gram-positive cells is not as well understood, although functional *oriT* sequences have been identified within the streptococcal and staphylococcal conjugative plasmids pIP501 and pGO1 respectively (52, 313), suggesting that conjugative systems in both Gram-positive and Gram-negative bacteria share a common transfer mechanism (108).

Non-self conjugative plasmids can be mobilised in *trans* by both conjugative plasmids and conjugative transposons. The basic requirement for mobilisation is the presence of an *oriT* and frequently a *mob* gene, which encodes a protein which specifically nicks the *oriT* site and is required to help form the relaxosome prior to

transfer (58). Other transfer functions are provided by the mobilising element. Non-self conjugative plasmids can also be mobilised in *cis* via the formation of a co-integrate with a conjugative element (255).

Fig 1.6: Plasmid conjugation from F+ cell to F- cell



From Firth *et al.* (87).

a) Cell-to-cell contact mediated by F-pili.

b) Mobilisation and transfer of a single stranded DNA to the recipient cell.

c) The double stranded DNA is nicked and one strand is transferred into the recipient cell.

d) The cells separate and both donor and recipient have a complete F plasmid and can conjugate with other F- recipient cells.

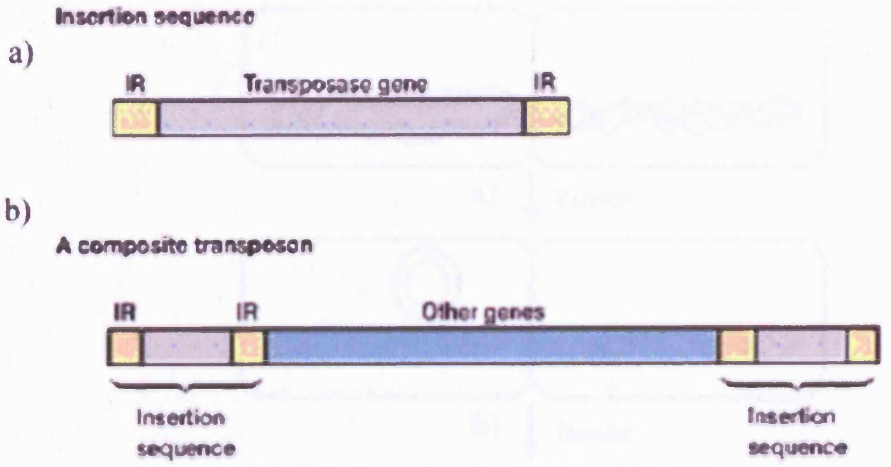
1.2.2.2.3.2 Transposons

Transposons are pieces of DNA which can copy and insert themselves at non-homologous regions of the genome. Unlike plasmids, transposons do not have the necessary machinery for autonomous replication and so depend on the replication-proficient vector molecule in which they integrate (plasmid or chromosomal DNA) for their replication. There are two types of transposition: replicative and non-replicative. The non-replicative transposition is a 'cut-and-paste' mechanism with the help of a transposase. First the transposase makes a double-stranded cut in the donor DNA at the ends of the transposon and makes a staggered cut in the recipient DNA. Each end of the donor DNA is then joined to the overhanging end of the recipient DNA. DNA polymerase fills in the short overhanging sequences. Replicative transposition occurs via a strand transfer reaction involving the nicked transposon and a target to generate a strand transfer intermediate. Replication of this intermediate resuts in duplication of the transposon and a co-integrate structure resolved by the resolvase enzyme. Non-conjugative transposition always creates a short direct repeat of the target sequence that flanks the transposon (53).

1.2.2.2.3.2.1 Insertion sequences (IS)

Insertion sequences are the simplest of the transposable elements; they are small and genetically compact (Fig 1.10). IS elements generally encode no functions other than those involved in their mobility, they consist of a recombinationally active DNA sequence which defines the ends of the element together with a transposase, which recognises and processes the ends (169). Any region of chromosomal DNA which becomes flanked by copies of the same IS can potentially become a compound transposon (Table 1.8) (236). These transposons consist of IS sequences and genes that encode antibiotic resistance or catabolic activities. Most of these mobile elements carry only one or two resistance loci (169).

Figure 1.7: Insertion sequence



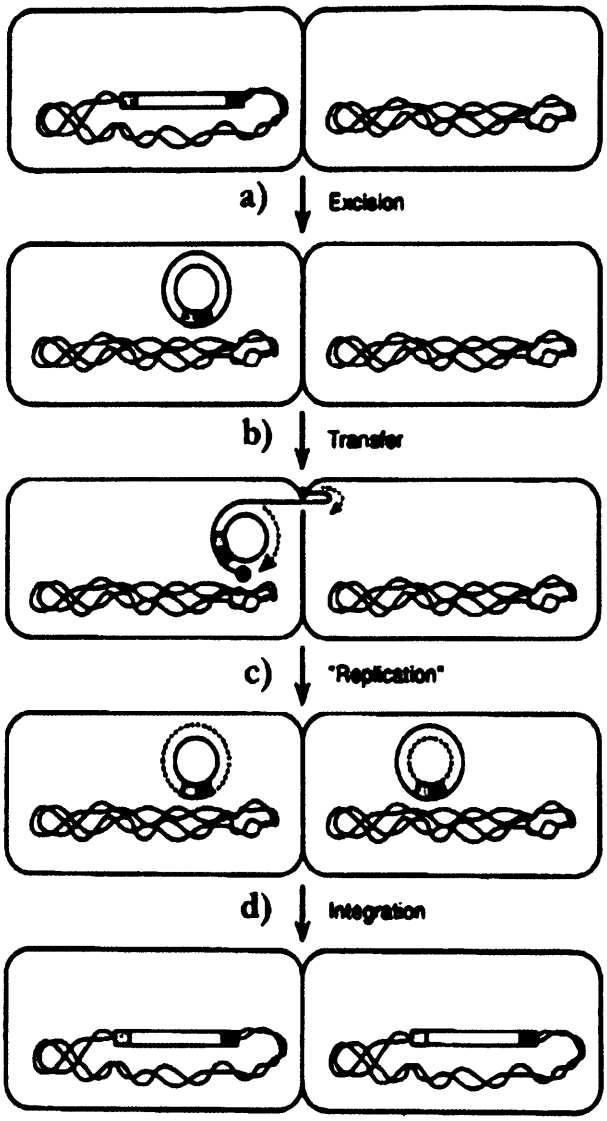
a- The central region of an IS element encodes a transposase and is flanked by inverted repeats (IR) elements that can vary in size between IS elements.
b- When a gene is flanked by two IS it is called a composite transposon.

1.2.2.2.3.2.2 Conjugative transposons

Some transposons are able to transfer only intracellularly, these are known as non-conjugative transposons whereas others are capable of transferring intracellularly and intercellularly, they are known as conjugative transposons. Conjugative transposons are common in Gram-positive and Gram-negative bacteria; some of them have a broad host-range (255).

These elements are plasmid-like in that they have a covalently closed circular transfer intermediate and are transferred by conjugation (Fig 1.7) but unlike plasmids the circular intermediate of a conjugative transposon does not replicate. Site specific recombinases encoded by these elements promote their excision and integration (183).

Fig 1.8: Example of conjugative transposon and the mechanism of transfer



From Salyers *et al.* (255).

This is an example of intercellular transposition of a conjugative transposon.

a) The conjugative transposon excises from the donor chromosome to form a circular intermediate.

b) A cleavage takes place at the *oriT* and a single strand DNA is transferred to the recipient cell by a mechanism similar to plasmid transfer (see chapter 1.2.2.2.3.1).

c) Replication from ssDNA to dsDNA in both the donor and recipient cells.

d) Integration of the dsDNA into the host chromosome.

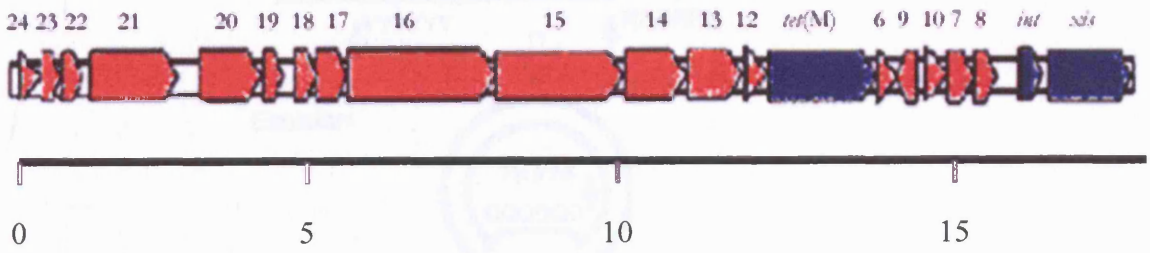
The closely related conjugative transposons Tn*916* and Tn*1545* have been found in a wide host range (51) including oral bacteria (143, 249). Tn*1545* is larger than Tn*916* and carries kanamycin and erythromycin resistance genes in addition to the tetracycline resistance gene *tet*(M), but its ends and most of its interior are virtually identical to the corresponding regions of Tn*916* (Fig 1.8). The excision-integration of Tn*916* is shown in Figure 1.9. Excision and integration require the integrase protein Int (a tyrosine recombinase) and the excisionase Xis (188). To excise the transposon, staggered nicks are generated by Int on each strand at 5 or 6 bp from the end of the element, these stretches of DNA are called coupling sequences (253). These coupling sequences often contain a heteroduplex however they are joined covalently to produce a circular intermediate (Fig 1.9). Prior to transfer to the new recipient cell, the double stranded circular form is nicked (at the *ori*T) and a single strand is transferred (255). Second strand synthesis occurs in the donor and recipient, the resulting double stranded molecule then integrates into the recipient and donor genomes. Other conjugative transposons were found to have different integration and/or excision systems; the bacteroides conjugative transposons CTnDOT containing *tet*(Q) use an integrase and a topoisomerase Exc instead of an excisionase Xis, Tn*5397*, closely related to Tn*916*, uses a serine recombinase TndX for its integration and excision (188).

Excision and transfer of Tn*916*, Tn*5397* and CTnDOT are regulated by tetracycline that leads to an increased expression of the transfer genes and thus an increased excision of the CTn (188, 323). Therefore antibiotics not only select for resistant strains but can also stimulate transfer of resistance genes.

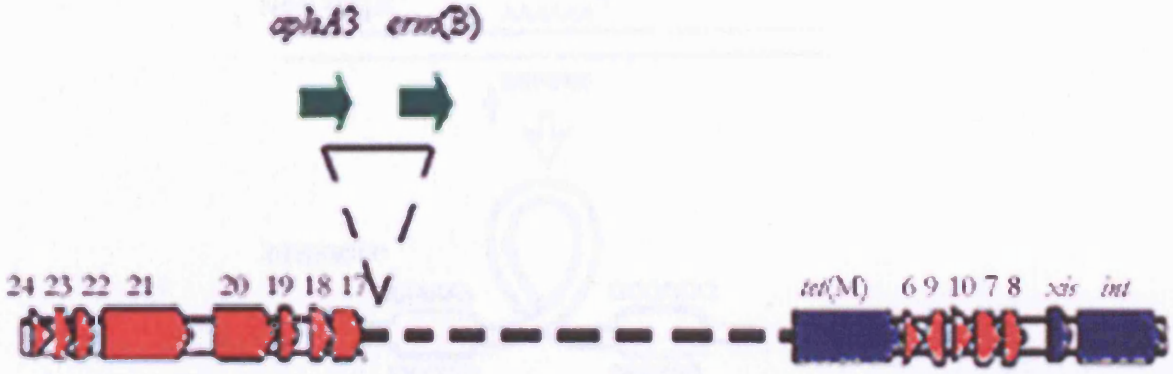
Unlike plasmids that are grouped into incompatibility groups according to their inability to stably coexist in a single host cell, conjugative transposons are sociable elements; they can coexist with each other in the same genome and they can physically associate with each other and with other genetic elements (Table 1.8) (300).

Figure 1.9: Comparison of Tn*916* and Tn*1545* family of conjugative transposons

a) Diagram of Tn*916* (18 kb)



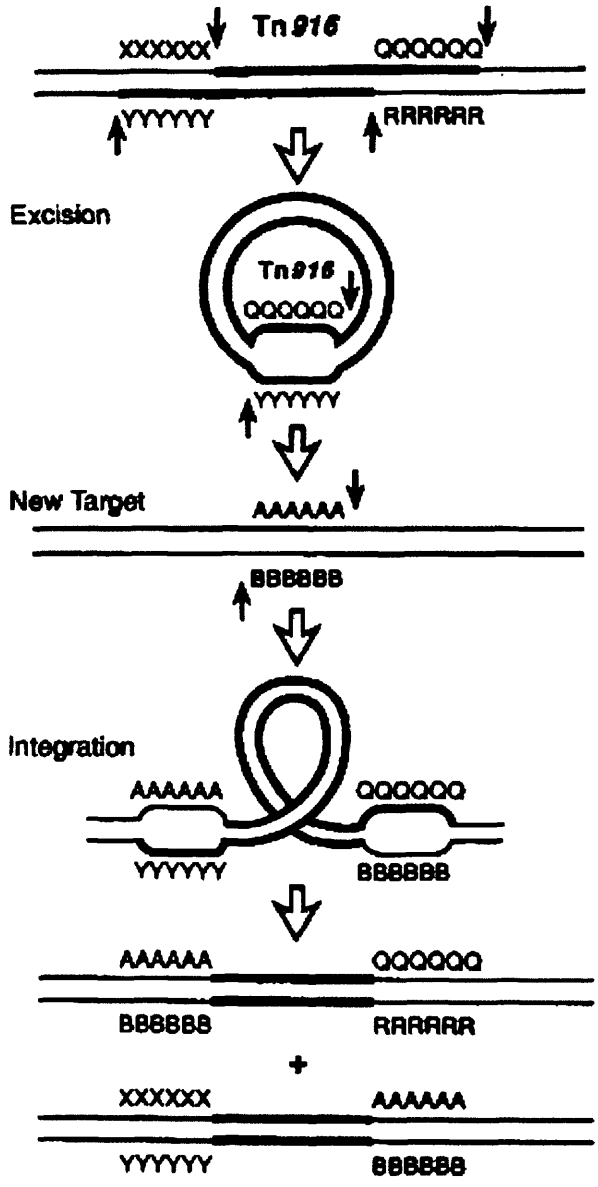
b) Diagram of Tn*1545* (25.3 kb)



a) The top diagram represents Tn*916*. The open reading frames of Tn*916* are represented by red and blue arrows showing the probable direction of transcription and are named as described in the work of Flannagan *et al.* (88). The bottom line is the scale in kilobases.

b) The green arrows represent the two extra antibiotic resistance genes (*aphA*3 and *erm*(B)) present in Tn*1545* according to Caillaud *et al.* (33) and the red and blue arrows represent the identical ends common in both Tn*916* and Tn*1545*, the arrows show the probable direction of transcription. The dashed black line represents the unknown sequence of Tn*1545*.

Figure 1.10: Excision and integration of Tn*916*



From Salyers *et al.* (255).

The transposon is represented by the thick line. The coupling sequences are represented by XXX/YYY or QQQ/RRR to indicate that initially they are complementary but QQQ does not pair with YYY. Staggered cuts, represented by the black arrows, occur 5 to 6 nucleotides from the ends of the element leading to the formation of single-standed overhangs (coupling sequences), which are joined covalently to produce the circular transposition intermediate. Staggered cuts open up the circular intermediate and its target site. Ligation produces a heteroduplex.

Table 1.8: Examples of transposons containing one or more functional module

Transposon [a]	Size (Kb)	Functional modules	Antibiotic resistance genes present	Ref
Tn*1545*	25.3	Tn*916*	*tet*(M) *erm*(B), *aphA*-3	51
Tn*3872*	21.6	Tn*916* Tn*917*	*tet*(M) *erm*(B)	174
Tn*3701*	>50	Tn*916* Tn*3703*	*tet*(M) *erm*(B)	147
Tn*5253*	65.5	Tn*5251* Tn*5252*	*tet*(M) *cat*[c]	12
Tn*1207.3*	52	Tn*1207.1*	*mef*(A)	259
Tn*2009*[a]	23.5	Tn*916*	*tet*(M) *mef*(E)	68
Tn*5385*	65	2 x IS*1216*[b] Tn*5381* + 2 x IS*1216*[b] Tn*5384* + 2 x IS*256*[b]	 *tet*(M) *Aac-6'-aph2*, *erm*(B), *mer*	236

[a] Most of the transposons listed are also conjugative except Tn*2009* that was shown to be transferred only by transformation (68).

[b] The transposon is flanked by two copies of an insertion element.

cat = chloramphenicol resistance gene, *aphA*-3 = kanamycin resistance gene, *Aac-6'-aph2* = aminoglycoside resistance gene, *mer* = mercuric chloride resistance gene.

1.2.2.2.3.2.3 Mobilisable transposons

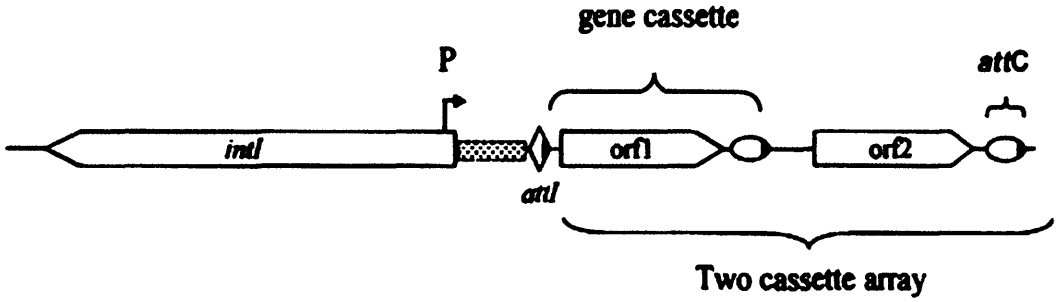
Like the conjugative plasmids, conjugative transposons can mobilise a coresident plasmid by providing the mating bridge through which the plasmid DNA transfers. Moreover, conjugative plasmids and conjugative transposons can also trigger the excision and circulation of unlinked integrated elements called mobilisable transposons (159, 255). So far, mobilisable transposons have been found only in *Bacteroides* (NBU, non-replicating *Bacteroides* units and Tn*4555*) and *Clostridium* species (Tn*4451*, Tn*4453*) (2). Mobilisable transposons are much smaller than conjugative transposons and contain genes required for excision, mobilisation and integration; however, they rely on transfer proteins supplied by coresident conjugative elements in order to transfer intercellulary, and on regulatory proteins that stimulate excision. Mobilisable transposons can become part of other mobile elements (324); they can also carry anbtibiotic resistance genes (2, 159).

1.2.2.2.3.3 Integrons

Integrons are composed of three key elements necessary for the procurement of exogenous genes: a gene coding an integrase (*intI*), a recombination site (*attI*) and a strong promoter (Fig 1.11). Integrons are able to capture genes from the environment and incorporate them by using site-specific recombination. The structural genes inside the integrons are assembled as cassettes, these gene cassettes are the smallest mobile elements known and include only one open reading frame (ORF) and a recombination site or *att*C site that is recognised by the integron-encoded integrase (*intI*) (54, 215). These gene cassettes can exist as free circular molecules, unable to replicate, or as part of an integron (231). Most of these gene cassettes do not contain a promoter, therefore their expression is dependent on integration in the correct orientation into an integron that supplies an upstream promoter adjacent to the *att*I site (54). The arrangement of the cassettes can be altered by excision of individual cassettes in the integrons or reassortment or new cassettes can be inserted (54). Over 60 cassettes have been identified (197), and most of them contain an antibiotic resistance gene, making these mobile elements a very efficient way of capturing and

disseminating antibiotic resistance genes and a very efficient mechanism by which plasmids and transposons acquire multiple antibiotic resistance determinants (231).

Figure 1.11: Structure of an integron



Adapted from Nield *et al.* (197).

Structure of an integron showing the positions of the integrase (*intI*), the recombination site (*att*I), the promoter (P) with the black arrow showing the direction of the promoter. This integron possesses two cassettes open reading frame 1 and 2 (*orf1* and *orf2*) with their own recombination site (*att*C).

Multidrug resistance in bacteria has been spreading alarmingly in the last decades (see chapter 1.2.1.1.3) and it is easy to understand why, considering the ways these genetic elements (IS, integron, CTn and plasmids) can acquire antibiotic resistance genes and spread them to a variety of different bacteria (254). Because these elements can also recombine with each other to generate mosaic mobile genetic elements (Table 1.8), their classification and nomenclature is becoming blurred, therefore they are often described as functional modules (32, 212, 300).

1.3 Antibiotic resistance in the oral cavity

The oral microbiota consists of over 700 species (326), although this figure is continually being revised upwards as molecular methods are being used to explore the diversity of the microbial communities inhabiting the complex ecosystem (216, 310). The majority are commensal bacteria while a minority are opportunistic pathogens that can cause systemic diseases when they are outside their normal environments (66, 162, 268, 292, 326). There is considerable evidence that the oral microbiota acts as a reservoir for antibiotic resistance genes and can pass these on to pathogenic bacteria (see chapter 1.3.2.2.2).

1.3.1 The oral microbiota

1.3.1.1 Oral bacteria organised in biofilms

The presence of nutrients, epithelial debris and secretions make the mouth a favourable habitat for a great variety of bacteria. Bacteria have colonised not only the teeth but the whole oral cavity including the tongue, the gingivae and between the gingivae and the teeth (321). Although the oral cavity provides some unique habitats for bacterial colonisation (teeth, mucosal surfaces, gingival crevices), the mouth has a resident microbiota which consists of many different species and genera. The oral streptococci comprise a large proportion of the resident microbiota and are isolated from all sites; they are classified in four groups (mutans, salivarius, oralis and milleri) and are commonly known as viridans streptococci. The oral microbiota also consists of *Staphylococcus* spp. that may be present transciently; *Actinomyces* spp. that are commonly found in dental plaques; *Neisseria* spp., low number are found from most oral sites; *Veillonella* spp., mostly located on the tongue surface and in dental plaque; *Haemophilus* spp., commonly found in saliva, dental plaque, and on epithelial surfaces, while *Eikenella*, *Porphyromona*, *Prevotella* and *Fusobacterium* spp. are commonly found in subgingival plaques (14).

Most of the bacteria in the oral cavity live in biofilms; they form complex dynamic communities on the surface of the teeth called dental plaque and are responsible for

inducing caries and periodontal diseases (3). Bacteria organised in biofilms have been shown to be 10-1000 times less susceptible to the effect of antimicrobial agents than their planktonic counterparts (312, 337). Multiple mechanisms are thought to be involved in this reduced susceptibility to antibiotics including the exopolymer matrix enclosing the biofilms, which can act as an ion-exchange matrix and impede charged antimicrobial agents (57, 168, 312, 337).

Since oral bacteria live in close proximity they are likely to exchange genes more readily. A donor containing a plasmid was introduced in both natural and laboratory-based biofilms and the experiment showed an initial rapid spread of the plasmid in the heterogenous bacterial communities (160, 184). Therefore the transient passing of cells carrying conjugative plasmids represents a potential source of spread of resistance genes to the indigenous oral microbiota. Furthermore, the recovery of Tn*916*-like elements in biofilms taken from the oral cavity (143, 249) and the transfer of Tn*916*-like elements in microcosm dental plaques between different oral streptococci (243) support the idea that oral bacteria are responsible for harbouring and disseminating mobile elements.

1.3.1.2 Antibiotic-resistant bacteria

The oral cavity is also exposed to the environment, so that the ingestion of food, water, a close contact with the surroundings and the consumption of medicines can all have an effect on the composition of the microbiota. A recent study has shown that in a model oral biofilm, the consumption of one antibiotic, tetracycline, had an effect on the whole microbiota; firstly altering its composition since the sensitive population decreased and was replaced with more resistant bacteria, and secondly by selecting not only for tetracycline-resistant bacteria but also bacteria resistant to other unrelated agents (230).

Oral bacteria have also been shown to carry a wide range of antibiotic resistance genes (248) and some of these are contained within conjugative DNA elements (245, 247, 249). Therefore there is clear evidence that oral bacteria are likely to play an important role in the development of resistance to antibiotics.

1.3.2 Evidence for gene transfer in oral and model biofilms

1.3.2.1 Gene transfer mechanism in oral bacteria

As described previously, bacteria can acquire antibiotic resistance genes by three different mechanisms: transformation, transduction and conjugation. There is evidence that all three take place in the oral microbiota.

1.3.2.1.1 Transformation

Members of the bacterial genera *Haemophilus*, *Neisseria* and *Streptococcus* are naturally competent for DNA uptake from their environment (163). The fact that most of the bacteria live in biofilms in the oral cavity is likely to facilitate this mechanism of exchange. Biofilm-grown *Streptococcus mutans* were transformed at a rate of 10- to 600-fold higher than planktonic *S. mutans*, and the dead cells in the biofilm were able to act as donors of a chromosomally encoded antibiotic- resistant determinant (158). Moreover, experimental evidence showed that *S. gordonii* could take up free extracellular DNA from saliva *in vitro* (181, 182). Genetic exchange between oral bacteria from two different genera, *Streptococcus gordonii* and *Treponema denticola*, in broth and artificial biofilms has been shown to take place by transformation (314).

1.3.2.1.2 Transfer of mobile elements

Transfer of transposons has been demonstrated in a microcosm dental plaque between different streptococcal species (243). Tn*916*-like elements are commonly found in the oral microbiota (19, 143, 249) and have been shown to transfer tetracycline resistance in a model oral biofilm. Transfer of elements located on mobilisable plasmids can also take place in a biofilm (184); rapid plasmid transfer occurred immediately after the introduction of a donor in a biofilm of *E. coli* (160). Although this last study was not performed in a model oral biofilm it underlines the potential role of transient cells, such as in the oral cavity, carrying conjugative

plasmids as a source of spread of resistance genes. Transfer of conjugative transposons between non-oral bacteria and oral commensals has been demonstrated in a mixed species oral biofilm. Tn*5397*, a conjugative transposon originally isolated from *Clostridium difficile* and conferring tetracycline resistance via *tet*(M), contained in a *B. subtilis* strain could be transferred within 6 h of inoculation of the donor to the oral commensal *S. acidominimus* (244). Thus, conjugative transposons can be transferred between bacteria from different environments.

1.3.2.1.3 Transduction

Examples of transfer by transduction are scarce; transduction has been shown to be responsible for the transfer of a chromosomal tetracycline resistance gene between strains of *S. aureus* in biofilm and liquid culture (301). However there has been no evidence of transduction in oral biofilms, and only few bacteriophages have been isolated from the oral cavity (119).

1.3.2.2 Implications of the presence of a pool of resistance genes in the oral cavity

1.3.2.2.1 Viridans streptococci and systemic diseases

Viridans group streptococci (VGS) are part of the indigenous microbiota of the upper respiratory tract of healthy humans. However, they have been associated with infective endocarditis, which most often results from the invasion of the bloodstream from the oral cavity as a result of poor dentition or extensive dental manipulation (66, 162). The emergence of strains intermediately resistant or highly resistant to penicillin, aminoglycosides, tetracycline and macrolides has been described worldwide (74, 218, 294) and is of concern since it not only limits the available options for the therapy of serious infections, but also increases the reservoir of antibiotic resistance genes that could be passed on to more pathogenic bacteria.

1.3.2.2.2 Gene exchange between commensal oral bacteria and pathogenic bacteria

:

Viridans group streptococci serve as a reservoir for many antibiotic resistance genes (19, 31, 120) and they have the potential to transfer their resistance genes to more pathogenic bacteria. The increased use of macrolides is thought to have led to the emergence of erythromycin resistance in *S. pneumoniae* and *S. pyogenes* (60, 83, 98, 173, 266), two important pathogens that share the same habitat as the VGS, that is the upper respiratory tract. Increased resistance of VGS to macrolides has also been observed and the genes conferring this were commonly found in VGS, *S. pneumoniae* and *S. pyogenes*. Particularly the *mef* gene, which encodes an efflux protein, was transferred from VGS to *Enterococcus faecalis* (165) and between *Streptococcus* spp. (259). One *mef* gene from an oral *Streptococcus* sp. had 100% identity at the DNA and amino acid levels with the *mef* gene found in *S. pneumoniae* (164); this is proof of gene exchange of this gene between these two species, either directly or via an intermediate organism. *Peptostreptococcus* spp., members of the pharyngeal, dental and gingival microbiota, have been found to harbour the *ermTR* gene (233), subsequently named *ermA* (247), which is also the most common methylase gene found in *S. pyogenes* strains (83). The methylase gene was transferred between these two species suggesting that this gene may circulate among both aerobic and anaerobic cocci of the oropharyngeal microbiota (233).

There is good evidence that the altered PBPs in pneumococci, leading to a decreased affinity for almost all β-lactam antibiotics, have emerged from the replacement of their normal PBP genes with those from penicillin-resistant oral streptococci such as *S. mitis* and *S. oralis* by transformation (232, 276). Recently fluoroquinolone resistance determinants were transferred *in vitro* between VGS and *S. pneumoniae* by transformation (128) as well as from clinical isolates of VGS to *S. pneumoniae* (105) and the mosaic structure of the genes conferring fluoroquinolone resistance in *S. pneumoniae* were shown to originate from VGS (15). Equally, penicillin resistance in the pathogenic strains of *N. meningitidis* and *N. gonorrhoeae* are thought to derive from the oral commensal species, *N. flavescens* and *N. cinerea* by transformation (276). Therefore, the indigenous oral microbiota appears to play an important role in the development of resistance to antibiotics and in the transfer of these genes to more pathogenic bacteria.

1.3.2.2.3 Evidence of antibiotic resistance gene transfer from transient organisms

In one study, the nucleotide sequence for the *tet*(Q) gene from a *Prevotella intermedia* strain, an oral pathogen, was compared to the sequences obtained for *tet*(Q) from *Bacteroides thetaiotaomicron*, a human intestinal organism, and for *tet*(Q) from *Prevotella ruminocola*, a rumen bacterium from cattle. Very high similarities (>=97%) were obtained among all three *tet*(Q) genes (311), indicating that natural horizontal transfer of tetracycline resistance has occurred between bacteria from the rumen of animals and bacteria from the human oral and intestinal environment, or that these genes may have transferred via an intermediate host.

There is overwhelming evidence that oral bacteria are a reservoir for different antibiotic resistance genes and that there is transfer of genetic material between different environments including the oral microbiota.

The euphoria produced by the discovery of antibiotics, and thus the belief that bacterial infections would become a thing of the past, quickly vanished with the emergence of antibiotic resistance both in hospital and in community settings. To make matters worse, bacterial pathogens have become increasingly resistant to a variety of antibiotics. The driving force behind the emergence of antibiotic resistance in bacteria is the ease with which bacteria can acquire resistance genes, even from distantly related genera combined with the selection pressure provided by the use of antibiotics. Commensal bacteria (including those present in the oral cavity) are thought to play an important part as a reservoir for antibiotic resistance genes.

Aims of the study

1) To determine the prevalence in the oral cavity of bacteria resistant to the following antibiotics: tetracycline, amoxycillin, erythromycin, gentamicin and vancomycin. The first three of these are the most frequently used antibiotics in dentistry (86) and in clinical practice in general, while concerns have been raised recently about the emergence of resistance to the last two antibiotics in pathogenic strains for which no other antibiotics are available.

2) To determine the nature, and prevalence, of tetracycline and erythromycin resistance genes contained in antibiotic-resistant bacteria.

3) To determine if the tetracycline and erythromycin resistance genes found are transferable and to investigate the genetic elements responsible for transfer.

Therefore this project will provide details of the prevalence of antibiotic resistance in the indigenous oral microbiota of humans as well as provide a better understanding of the mechanisms of resistance.

Chapter 2

Materials and Methods

2.1 Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Appendix 1.

2.2 Sampling and processing of samples

2.2.1 Subjects

Three batches of 20 healthy adults aged 16-45 years referred to the Orthodontic Department at the Eastman Dental Hospital were invited to participate in the study. The patients had not received antibiotics within the last three months and represented a cross-section of people from different ethnic groups, gender and age.

2.2.2 Sample collection and processing

For each patient, a saliva sample was obtained by expectoration into a sterile container. Plaque samples were collected using a calcium alginate swab (Technical Service Consultants, Heywood, UK) and subgingival plaque samples were collected from four different sites with sterile paper points; both samples were pooled into 4 ml of Calgon Ringer's solution (Oxoid, Basingtoke, UK) in a sterile bijou containing five sterile 2 mm diameter glass beads (BDH Chemicals, Poole, UK). The samples were vortexed for 30 s to dissolve the calcium alginate and mixed with the saliva sample for each individual. A 10-fold serial dilution of the sample was then prepared in tryptone soya broth (Oxoid) and spread onto both antibiotic-containing and antibiotic-free agar plates (to determine the total number of cultivable bacteria in the specimen) in duplicate (petri dishes from Sarstedt). Iso-sensitest agar (Oxoid) supplemented with 5% defibrinated horse blood (E&O Laboratories, Bonnybridge,

UK) was used and amoxycillin (Sigma-Aldrich, Poole, UK), erythromycin (Sigma-Aldrich), gentamicin (Sigma-Aldrich), tetracycline (Sigma-Aldrich), vancomycin (Sigma-Aldrich) at a breakpoint concentration of 1, 1, 4, 8, 8 μg/ml respectively, based on the recommendations of the NCCLS (192). Stock solutions of each antibiotic were prepared monthly at a 1000-fold greater concentration than that used for selection. Gentamicin and vancomycin were reconstituted in distilled water, erythromycin in 95% ethanol, tetracycline in 50% ethanol/50% distilled water. Amoxycillin was reconstituted in 0.01 M potassium phosphate buffer, pH 7. All antibiotic stock solutions were filter- sterilised and stored at - 20°C.

Staphylococcus aureus NCTC 6571 was used as the quality control organism.

One set of plates was incubated in an anaerobic chamber (MACS 1000, Don Whitley Scientific, Shipley, UK) for 7 days at 37°C and a duplicate set was incubated in air supplemented with 5% $C0_2$/air for 2 days at 37°C.

2.2.3 Enumeration and storage

After incubation, the resistant isolates were enumerated according to their morphology and one representative colony of each colony type was subcultured and incubated under both aerobic and anaerobic conditions to ascertain its atmospheric requirements. All obligate aerobes were then subcultured and stored in Microbank™ (Pro-Lab Diagnostics) and kept at –70°C. Colonies with different morphologies on the anaerobic plates were also counted separately; these were then subcultured and stored at -70°C (Microbank).

2.2.4 Identification of antibiotic-resistant bacteria

Preliminary identification of the isolates was carried out on the basis of atmospheric growth requirements, Gram-stain (from Pro-Lab Diagnostics UK), catalase (using Hydrogen Peroxide from Sigma) and oxidase (N, N, N', N'-Tetramethyl-P-Phenylenediamine from Sigma) reactions. Additionally, a number of strains were

further characterised, and in most cases identified to the species level, using partial 16S ribosomal RNA (rRNA) gene sequencing (145). The 16S rRNA was amplified using the primers 27F (5' AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGA CTT-3') (Genosys, Sigma, UK) (145). Subsequent partial DNA sequencing of the 16S rRNA gene was carried out using a single primer 357F (5'-CTCCTACGGGAGGCAGCAG-3') and an ABI310 Genetic Analyser (PE Biosystems, Warrington, UK).

2.2.5 Antibiotic susceptibility testing

The MIC of each isolate for amoxycillin (Sigma-Aldrich, Poole, UK), erythromycin (Sigma-Aldrich), gentamicin (Sigma-Aldrich), tetracycline (Sigma-Aldrich) and vancomycin (Sigma-Aldrich) was determined by an agar dilution assay on iso-sensitest agar plates (Oxoid) supplemented with 5% defibrinated horse blood. The inoculum was standardised using a 0.5 Macfarland standard in accordance with NCCLS recommendations (192) and inoculated onto the agar using a multi-point inoculator (Mast Diagnostics, Bootle, UK) and were read after 24 h incubation at 37°C aerobically or 48 h incubation at 37°C anaerobically according to the atmosphere requirement of the isolate. The highest dilution of antibiotic that inhibited visible growth was taken to be the MIC. *Staphylococcus aureus* NCTC 6571 and Iso-sensitest broth (Oxoid) were included on the plates as controls.

2.2.6 Statistical Analysis

The outcome measure to be analysed statistically was the proportion in each of the three patient groups of resistant isolates in the oral microbiota for each antibiotic. The analysis was carried out using the Kruskal-Wallis test using SPSS software (SPSS Inc. (2000). SPSS 10.0 Syntax Reference Guide. Chicago, IL). Any differences in the proportion of resistant bacteria for each antibiotic between each patient group were assessed using the chi-square analysis. A P value <0.05 was considered significant.

2.3 Extraction of genomic DNA

DNA from Gram-positive and Gram-negative bacteria was extracted according to the Gram-positive bacteria protocol from the Puregene Kit (Flowgen, Gentra System). A pure culture of each organism was grown overnight at 37°C on a selective blood agar plate. The colonies were suspended in 1 ml of sterile distilled water in an eppendorf tube (Sarstedt) using a swab (BDH). The cells were pelleted by centrifugation at 15,800 g in a bench top microcentrifuge (Jouan A14) for 1 min and resuspended in 300 µl of Cell Suspension Solution provided by the kit. A volume of 1.5 µl of Lytic Enzyme Solution (4,000 U/ml) was added; the samples were inverted 25 times and incubated at 37°C for 30 minutes (Grant incubator) to digest cell walls. After centrifugation of the samples for 1 min at 14,000 rpm (g force of 15,800) the supernatant was removed and the cells were suspended in 300 µl of Cell Lysis Solution provided by the kit (Puregene) and gently pipetted up and down to lyse the cells. The samples were heated at 80°C for 5 minutes to complete cell lysis. A volume of 1.5 µl of RNAse A Solution (4 mg/ml) was added to the cell lysate, the samples were mixed by inverting the tubes 25 times and then incubated at 37°C for 1 h to denature the RNA. After cooling the samples to room temperature, 100 µl of Protein Precipitation Solution (Puregene) was added to the cell lysate and incubated on ice for 30-60 minutes. The samples were centrifuged for 3 minutes at 14,000 rpm (g force of 15,800) and the supernatant containing the DNA was poured into a clean 1.5 ml eppendorf tube containing 100% Isopropanol (Sigma). The samples were inverted gently 50 times and centrifuged for 1 minute at 14,000 rpm (g force of 15,800). After removing the supernatant, the DNA pellet was washed with 300 µl 70% Ethanol (100% AnalaR BDH, diluted with sterile distilled water) and centrifuged for 1 minute at 14,000 rpm (g force of 15,800). The tubes were drained and allowed to air dry for 30 minutes. The DNA pellet was redissolved overnight in a suitable volume (100 µl) of DNA Hydration Solution (Puregen) or sterile water.

Some samples yielded too low a DNA-concentration and so the DNA extraction procedure was modified. After overnight incubation on a selective blood agar plate, a loopful of pure colonies was suspended into a universal (Sarstedt) containing 5 mls of brain heart infusion broth or BHI (Oxoid) and incubated on a shaker (Sanyo, orbital incubator) overnight at 37°C. The cells were centrifuged for 6 min at 5,000

rpm (Eppendorf, 5804R, g force of 4500) and the pellet was suspended in 300 µl of Cell Suspension Solution (Puregen) transferred in an eppendorf tube and 30 mg/ml of lysozyme solution (Sigma) was added. The samples were incubated at 37°C for 1 h centrifuged for 1 min at 14,000 rpm (g force of 15,800) and the pellets were resuspended in 300 µl of Cell Lysis Solution (Puregene); then the same procedure as before was followed.

2.4 Basic/16S rRNA PCR protocol

Genomic DNA was extracted and purified as noted earlier. The subregion of the 16S rRNA gene amplified was 1465-bp sequences between primers 27F and 1492R. In a total reaction volume of 50 µl, 0.1 volume of buffer (Bioline) was mixed with 3 µl of $MgCl_2$ (50 mM from Bioline), 1 µl of dNTPs mixture (100 mM from Bioline), 2 µl of each primer (25 pmole), 2 µl of template (to a final concentration of 0.1 to 1 µg), 31.8 µl of sterile distilled water and 0.16 µl of Taq polymerase (Bioline). PCR were performed for 29 cycles of 60 s at 94°C, 60 s at 54°C, and 90 s at 72°C, with a final extension at 72°C for 5 min (Primus PCR machine).

2.5 Agarose gel electrophoresis

The genomic DNA, plasmid samples, PCR products were analysed by agarose (Amresco, Solo, Ohio, USA) gel electrophoresis (256). The gels were made up with 1 x TAE (Appendix 2) according to the volume needed. The mixture was heated in a microwave (Sharp Compact) and after cooling, 0.5 µg/ml of Ethidium Bromide (Sigma) was added and the agarose was poured into the appropriate tank.

Samples were mixed with 0.5 Volume of Blue/Orange Loading dye, 6X (Promega) and loaded into the gel. HypperLadderII (Bioline) was used as a marker unless specified and 5 µl was loaded in parallel with the samples. The gels were run at an appropriate voltage for an appropriate length of time depending on the size of the DNA to be visualised.

The gel was exposed to UV light to visualise the DNA (Alpha Innotech Corporation, distributed through Flowgen, Ashby de la Zouch, UK) and a picture of each gel was taken (Alpha Imager™ 1220 Documentation and Analysis System) saved and printed.

2.6 Purification of PCR samples

Prior to sequencing, the PCR products were purified from the other components in the reaction such as excess primers, nucleotides, DNA polymerase, oil and salts using the GenElute™ PCR Clean-Up Kit (Sigma). Each sample (50 µl) was mixed with 5 volume of Binding Solution (250 µl), transferred into a miniprep column and centrifuged for 1 min at 14,000 rpm (g force of 15,800). The flow-through was discarded and 500 µl of diluted Wash Solution (12 ml of Wash Solution diluted with 48 ml of Ethanol 100%) was added, the sample was centrifuged for 1 min at 14,000 rpm (g force of 15,800). The flow-through was discarded and the sample was centrifuged again for 2 min. The column was transferred to a fresh eppendorf tube, 50 µl of Elution Buffer was applied to the centre of the column and each tube was incubated at room temperature for 1 min. To elute the DNA, the column was centrifuged for 1 min. The sample was run on a gel to confirm the presence of DNA before sequencing.

2.7 DNA sequencing

The sequencing of PCR products was carried out according to the PE Biosystems (Warrington, UK) instructions with the following modifications. In a total volume of 7 µl, 5 pmol of primers was mixed with 2 µl of 1:4 diluted ABI BigDye Terminator Ready Reaction Mix, and 1 to 4 µl of DNA sample. The samples were then run on the following program; rapid thermal ramp to 95°C, held for 10 sec, rapid thermal ramp to 50°C, held for 5 sec, rapid thermal ramp to 60°C, held for 4min. These four steps are repeated for 99 cycles followed by a rapid thermal ramp to 4°C and held until the samples were ready to purify.

2.7.1 Purification of sequencing products

To the PCR tubes was added 15 µl of H_2O, 2 µl of 3M NaAcetate (Sigma) and 50 µl -20°C 100% ethanol (100% AnalaR BDH) and then incubated on ice for 20 min. The samples were spun at 14 K (g force of 15,800) for 25 min, the supernatant was discarded and the pellet washed and centrifuged with 250 µl -20°C 70% ethanol for another 15 min at 14 K (g force of 15,800). The supernatant was removed and the sample was dried at 95°C for a few seconds and resuspended in 20 µl of template suppresser reaction buffer (Applied Biosystems). The samples were then loaded on the ABI PRISM® 373 DNA Sequencer according to the manufacturer's instructions and the results were retrieved from the database and saved onto discs for further analysis.

2.7.2 Analysis of sequencing products

The sequence data obtained where compared to those in public sequence repositories (GenBank) using the basic local alignment search tool (BLAST) (6).

2.8 Filter-mating

Donor and recipient were grown separately on Iso-sensitest agar plates (Oxoid) containing relevant antibiotics at 37°C overnight. Each isolate was suspended in 5 ml of Brain Heart Infusion broth (Oxoid) to a density of approximately 10^9 cells per ml (3 McFarland units). Donor and recipient bacteria in the ratio of 1:5 (donor to recipient) were mixed. The bacterial suspension was then spread on 0.45 µm nitrocellulose filters (Fisher, Scientific, London, UK) and incubated at 37°C for 24 h. After incubation, the mixture was resuspended in 2 ml of BHI broth; the filters were removed and placed in a sterile universal containing the broth and vortexed for 10 s. 100 µl of the mixture was spread on antibiotic-supplemented plates. These were incubated for up to 48 h at 37°C. Transconjugants were tested for the presence of specific antibiotic resistance genes by PCR and hybridisation as well as by partial

16S rRNA gene sequencing to rule out the presence of a donor mutant. The transfer frequency of each mating was calculated as the rate per donor CFU.

2.9 Southern blotting

Southern blotting was performed using the kit from Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, England.

2.9.1 Restriction enzyme digests

All the samples were digested from genomic DNA with *Hin*dIII (Promega) at 37°C for 2 h. For the digestion reaction 14 µl of DNA (at a concentration in between 10 to 50 µg/ml) was mixed with 2 µl of Buffer, 2 µl of enzyme (10 U/µl) and 2 µl of distilled H_2O for a total volume of 20 µl. The whole sample was loaded into a 100 ml 1% agarose gel (as described in chapter 2.5) and run overnight at 20 mV. Lambda DNA BstE II Digest (New England Biolabs) was used as the marker.

2.9.2 Processing the Gel

After running, the gel was placed in a suitable container covered with fresh depurination solution (Appendix 2) and agitated until the bromophenol blue dye turned completely yellow. The depurination solution was discarded and the gel was rinsed with distilled water. The gel was then covered with denaturation solution (Appendix 2) and agitated for 25 min after the bromophenol blue dye had turned back to the original blue color. The gel was rinsed again with distilled water and covered with neutralization solution (Appendix 2) and agitated for 30 min.

2.9.3 Capillary Blotting

The capillary blotting consisted of a pyrex dish filled with 20 x SSC (Appendix 2), one sheet of Whatman 3MM saturated with 20 x SSC and covering a supporting platform. The gel was placed on the 3MM paper, taking care to avoid trapping air

bubbles and surrounded with cling film to prevent the SSC being absorbed directly by the paper towels. A sheet of Hybond N+ nylon membrane was cut to the exact size of the gel and placed on top of the gel making sure not to trap any air bubbles. Three sheets of absorbent filter paper (Merck, Lutterworth, UK) cut to the size of the gel and soaked in 10 x SSC (20 x SSC diluted 1 in 2 in distilled water) were placed on top of the membrane, again avoiding trapping air bubbles. A 5-7 cm stack of absorbent paper towels were placed on top of the filter paper. This was left overnight at room temperature.

2.9.4 Processing the blot

The blotting stack was dismantled and the membrane and gel removed together. The membrane was peeled off from the gel and placed on a fresh piece of filter paper DNA side up. The DNA side was marked with a pencil. The membrane was placed in 6 x SSC (20 x SSC diluted 1 in 3 in distilled water) and rinsed for 1 min with gentle agitation to remove any agarose. The membrane was then placed on another fresh piece of paper and the DNA fixed by UV cross-linking in a UV Stratalinker 1800 (Stratagen Europe, Amsterdam, Netherlands) according to the manufacturer's instructions.

2.9.5 ECL Direct Nucleic Acid Labeling and Detection System

2.9.5.1 Labeling of DNA probes

The DNA to be labelled was diluted to a concentration of 10 ng/μl using sterile distilled water. The DNA (100 ng in 10 μl) was denatured by boiling in a water bath for 5 min followed immediately by 5 min incubation on ice. The contents of the tube were then briefly spun to collect them in the bottom of the tube. An equivalent volume (10 μl) of DNA labeling reagent (Amersham Pharmacia Biotech) was added to the cooled DNA and mixed gently but thoroughly. This was followed by addition of the gluteraldehyde solution (Amersham Pharmacia Biotech) to the same volume as the DNA labeling reagent (10 μl) and mixed thoroughly. The contents of the tube

were again spun. The tube was incubated for 10 min at 37°C and, if not used immediately was held on ice for 10-15 min.

2.9.5.2 Hybridisation and stringency washes

The hybridisation buffer was prepared as followed; solid NaCl was added to the required volume of hybridisation buffer (50 ml) to a final concentration of 0.5 M. Blocking reagent was then added to a final concentration of 5% (w/v) and immediately mixed until the blocking reagent was present as a fine suspension. Further mixing with stirring for 1 h at room temperature was followed by incubation at 42°C for 0.5-1.0 h with occasional mixing. If the buffer was not being used immediately it could be stored at -20°C for 3 months.

2.9.5.3 Pre-hybridisation using tubes

The hybridisation buffer was preheated to 42°C. The blot was placed in a suitable container with 5 x SSC (20 x SSC diluted 1 in 4 in distilled water). The blots were loosely rolled into a tube and placed inside the hybridisation tube. The 5 x SSC was poured off and the appropriate amount of hybridisation buffer added (0.0625 – 0.125 ml/cm^2), the blot was pre-hybridised for 2 h at 42°C in a Biometra (Luton, UK) OV3 rotisserie oven and then the labelled probe was added to the buffer, taking care not to place it directly on the membrane. Hybridisation was carried out overnight in the rotisserie oven at 42°C. The appropriate volume of primary wash buffer containing urea (Appendix 2) was warmed to 42°C. The hybridisation buffer in the tube was discarded and 50-100 ml of 5 x SSC added. The tube was replaced in the rotisserie oven and incubated for 20 min at 42°C. The primary wash buffer was discarded and replaced with an equal volume of fresh primary wash buffer. This was replaced into the rotisserie oven and incubated for 10 min at 42°C. This wash was repeated for a second time. The blot was then removed from the hybridisation tube and placed in a suitable container. This was covered with an excess of secondary wash buffer 2 x SSC (20 x SSC diluted 1 in 10 in distilled water) and incubated at room temperature with gentle agitation on a rotary shaker for 5 min.

2.9.5.4 Signal generation and detection

An equal volume of ECL reagent 1 and ECL reagent 2 (Amersham) were mixed in a 20 ml universal tube. The amount mixed was just enough to cover the blot. The excess secondary wash buffer was drained from the blot and the blot was placed DNA side up onto a clean piece of cling film (Saran Wrap). The detection reagents were poured onto the blot immediately and incubated at room temperature for 1 min with gentle agitation. The excess detection reagents were drained from the blot and it was wrapped in cling film (Saran Wrap). Air bubbles were smoothed out and the blot was placed in a film cassette DNA side up. Autoradiography film (Hyperfilm, Amersham) was placed over the blot. The film cassette was closed and the film exposed for 1 to 3 min. The film was removed under safe light conditions and developed.

2.10 Cloning

2.10.1 Plasmid extraction

pUC18 was used as the plasmid of choice for cloning because of its high-copy number. Prior to extraction one colony containing pUC18 was inoculated in 5 ml of LB (Appendix 2) containing 50 μg/ml of ampicillin, and incubated 37°C with shaking overnight at 225 rev/min (Stuart Scientific Orbital Shaker SO1). Bacteria were recovered by centrifugation at 5000 rpm (g force of 4500) for 5 min and the plasmid was extracted using the miniprep kit (Qiagen) according to the manufacturer's instructions.

2.10.2 Plasmid digestion and dephosphorylation

pUC18 was digested with the most suitable restriction enzyme in a total volume of 100 μl. The subsequent dephosphorylation was done at 37°C for one hour by adding 1 unit (U) of alkaline phosphatase (Promega) at time 0 and 30 min. The mixture was inactivated with the addition of 2 μl of 0.5M EDTA (Appendix 2), incubated at 60°C

for 20 min. The sample was purified using GenElute™ PCR Clean-Up Kit (Sigma) and the final mixture was resuspended in 30 µl of elution buffer (10 mM Tris HCl pH 8.5) provided by the kit.

2.10.3 Gel extraction

This was performed using the QIAquick Gel Extraction kit (Qiagen Ltd, West Sussex, UK) following the manufacturer's instructions.

2.10.4 Ligation

Both the insert (gel extraction product) and the vector (dephosphorylated pUC18) were mixed together in a total volume of 100 µl including 9 U of T4 DNA ligase (Promega) and 10 µl of T4 DNA ligase buffer 10x (Promega). The sample was incubated overnight at 15°C and then at 60°C for 20 min to inactivate the reaction.

2.10.5 Transformation

50 µl of the ligation mixture was mixed with 200 µl of chemically competent cells (DH5α from Invitrogen). The sample was incubated on ice for 1 hr, heated at 42°C for 1 min. Then the cells were grown in 1 ml of SOC medium (Appendix 2) at 37°C for 90 min with constant shaking. The mixture was plated onto selective LB plates (Appendix 2) containing 50 µg/ml of ampicilllin and 5 µg/ml of tetracycline, and they were incubated for 18 h at 37°C.

2.11 Oligonucleotide Synthesis

The synthesis of all oligonucleotides was carried out by Genosys Biotechnologies (Europe) Ltd (Pampisford, UK). Sequences of all primers used throughout this study are shown in Appendix 3.

Chapter 3

Prevalence of antibiotic-resistant cultivable oral bacteria in healthy adults

3.1 Abstract

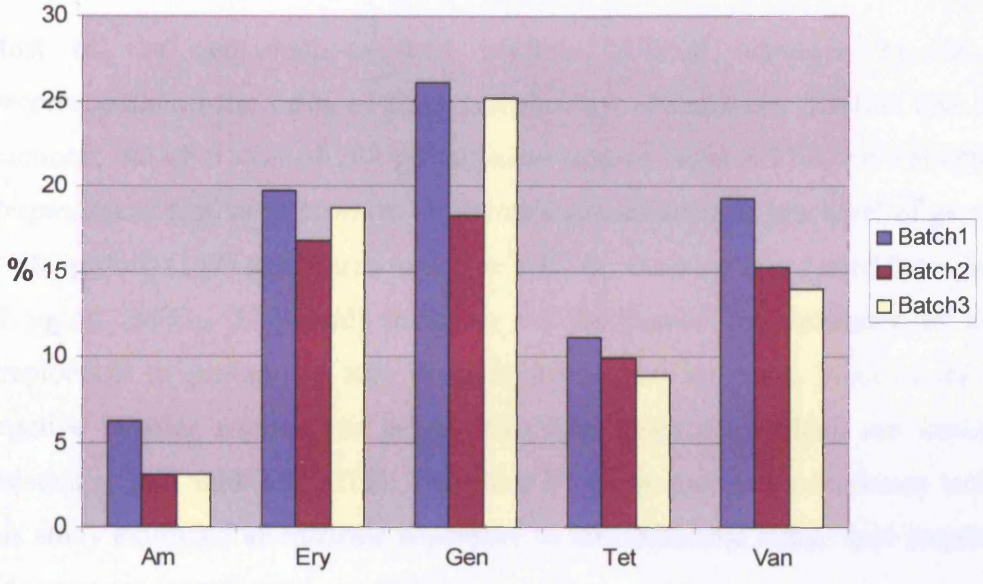
Oral bacteria, as well as causing caries and periodontal diseases, are also responsible for a number of life-threatening diseases (endocarditis, brain abscess and lung infection) for which antibiotics are required (66, 162, 268, 292). Although concern has been expressed about the rise in the prevalence of antibiotic-resistant bacteria, there is little information concerning their prevalence in the oral cavity. The aim of this chapter was to determine the prevalence of antibiotic-resistant oral bacteria in healthy adults. Saliva and plaque samples were obtained from 60 healthy adults (in three batches of 20 samples) who had not taken antibiotics during the previous three months. Each sample was plated onto media containing the following antibiotics: amoxycillin, erythromycin, gentamicin, tetracycline, vancomycin, and then incubated anaerobically and in 5% CO_2/air. Resistant isolates were enumerated and identified. All of the individuals were found to harbour bacteria resistant to erythromycin, gentamicin, tetracycline and vancomycin. Only 4 individuals did not have any cultivable bacteria resistant to amoxycillin. Oral bacteria resistant to gentamicin were the most commonly isolated followed by erythromycin, vancomycin, tetracycline and amoxycillin.

3.2 Results

Out of the 60 samples screened all of the individuals were found to harbour bacteria resistant to erythromycin, gentamicin, tetracycline, vancomycin and 4 individuals only (6.7%) did not have any cultivable bacteria resistant to amoxycillin.

From the 3 groups of patients studied, oral bacteria resistant to gentamicin were the most commonly isolated (a mean of 23.2% of the total viable count) followed by erythromycin (a mean of 18.4% of the total oral count), vancomycin (a mean of 16.1% of the total viable count), tetracycline (a mean of 9.9% of the total viable count) and amoxycillin (a mean of 4.1% of the total viable count) (Fig 3.1).

Figure 3-1: Proportion of antibiotic-resistant isolates expressed as a percentage of the total viable count



Am = amoxycillin, Ery = erythromycin, Gen = gentamicin, Tet = tetracycline, Van = vancomycin.

To establish whether the prevalence of antibiotic-resistant oral bacteria varied between the different groups, the 3 batches were compared using the Kruskal Wallis test. The difference in the proportion of resistant bacteria for each antibiotic and each batch was considered significant when the P value < 0.05. There was no significant difference found for the proportion of any of the antibiotic-resistant isolates between the three batches (Table 3.1).

Table 3-1: Comparison of the three batches of samples obtained from the three patient groups using the Kruskal-Wallis test

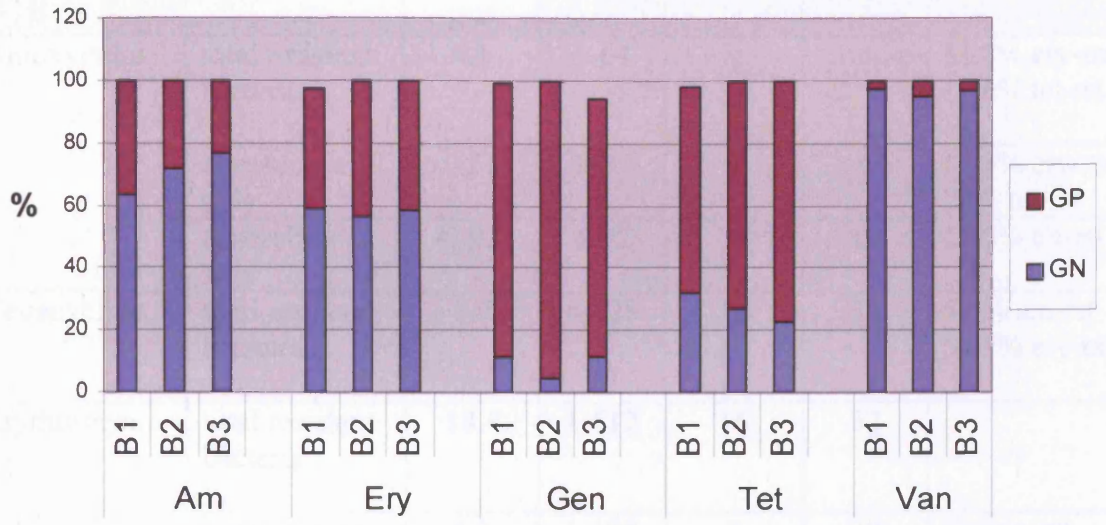
Antibiotic	*P* value[a]
Amoxycillin	0.54
Erythromycin	0.74
Gentamicin	0.36
Tetracycline	0.64
Vancomycin	0.37

[a] *P* value < 0.05 was considered significant

Most of the gentamicin-resistant bacteria isolated belonged to the genus *Streptococcus* on the basis of their morphology, Gram-stain, catalase and oxidase reactions; out of a total of 288 gentamicin-resistant isolates 93% were streptococci. *Streptococcus* spp. are known to be intrinsically resistant to low level of gentamicin (< 500 μg/ml) (167) and in this study the MIC for these species varied between 4 and 32 μg/ml (MIC_{90} 32 μg/ml) therefore the mechanism of resistance of the oral streptococci to gentamicin was intrinsic rather than acquired. Most of the Gram-negative isolates resistant to gentamicin were anaerobes, which are intrinsically resistant to this antibiotic (214). Therefore all of the gentamicin-resistant isolates in this study exhibited an intrinsic resistance to this antibiotic rather than acquired and thus were not investigated any further.

Most of the vancomycin-resistant isolates were Gram-negative bacteria (Fig 3.2) that are known to be intrinsically resistant to this antibiotic and all of the vancomycin resistant Gram-positive species were identified as *Lactobacillus* spp. by 16S rRNA sequencing, which are also known to be intrinsically resistant to vancomycin (91). Therefore from the 60 samples screened no bacteria containing acquired resistance to vancomycin were isolated.

Figure 3-2: Proportion (of the total viable count) of Gram-positive and Gram-negative resistant isolates for each batch and for each antibiotic



GP = Gram-positive, GN = Gram-negative, B1 = Batch1, B2 = Batch2, B3 = Batch3

Amoxycillin-resistant bacteria were isolated from 56 out of the 60 samples (93.3%). The median percentage was 4.1%, this ranged from 0% to 34.3%. A total of 224 amoxycillin-resistant bacteria were isolated, the MIC of amoxycillin of these isolates ranged from 1 µg/ml to 64 µg/ml. The majority (81.8%) of these amoxycillin-resistant isolates were also found to be resistant to erythromycin, 17.6% displayed resistance to tetracycline and 17% were resistant to both tetracycline and erythromycin (Table 3.2).

Table 3-2: MIC range, MIC_{50} and MIC_{90} values for amoxycillin-, tetracycline- and erythromycin-resistant bacteria isolated from the three groups of patients

Antimicrobial agent	Isolates	Median Value (%)	MIC range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	Multi-drug resistance
Amoxycillin	total resistant bacteria	4.1	1-64	1	4	81.8% ery-rst 17.6% tet-rst
	streptococci only	0.53	1-32	2	4	54.3% ery-rst 20% tet-rst
	anaerobes only	0.92	1-32	4	16	25.5% tet-rst
Tetracycline	total resistant bacteria	9.9	4-128	16	32	5.6% am-rst 54.5% ery-rst
Erythromycin	total resistant bacteria	18.4	1-512	16	32	
	Gram-positive only	6.3	1-128	8	128	3% am-rst 34.8% tet-rst

ery-rst = erythromycin-resistant, tet-rst = tetracycline-resistant, am-rst = amoxycillin-resistant.

Out of the 60 samples screened, all of the individuals were found to harbour tetracycline-resistant bacteria. The median percentage was 9.9%, this ranged from 0.2% to 38.9%. A total of 318 tetracycline-resistant bacteria were isolated, the MIC of tetracycline to these isolates ranged from 4 μg/ml to 128 μg/ml (Table 3.2).

The data for the erythromycin-resistant bacteria require careful interpretation because many Gram-negative bacteria are intrinsically resistant to this antibiotic or they can exhibit resistance through the presence of an efflux pump *mtr* (multiple transferable resistance) that is not specific to macrolides (187, 334). To gain a better idea of the occurrence of erythromycin-resistant bacteria in the oral cavity, the mean average was re-calculated with Gram-positive isolates only. From the 3 batches, a mean average of 6.3% of the total viable count was found to be resistant to erythromycin. Out of 129 erythromycin-resistant Gram-positive isolates, 3.1% (4 isolates) were also

resistant to amoxycillin and 34.8% (46 isolates) were resistant to tetracycline (Table 3.2).

3.3 Discussion

The total anaerobic count from the 60 samples ranged from 1.8 x 10^6 to 4.1 x 10^8 colony forming units (cfu) per ml of pooled saliva and plaque samples (mean = 7.4 x 10^7 cfu) and these results are similar to previous studies; two of which looked at the total viable count in the denture plaque of healthy adults (327), while two studies looked at the total viable count in subgingival plaque samples obtained from periodontal disease patients (113, 143).

In this study, the prevalence of different antibiotic-resistant bacteria in the oral cavity of a healthy adults organised in 3 batches of 20 samples each was compared. There was no significant difference between the batches when considering the proportion of gentamicin-, vancomycin-, amoxycillin-, erythromycin- and tetracycline-resistant oral bacteria. However, all of the vancomycin-resistant isolates were intrinsically resistant to this antibiotic and most of the gentamicin-resistant isolates were *Streptococcus* spp. with low level resistance (MIC_{90} 32 μg/ml). *Streptococcus* spp. are known to be intrinsically resistant to low level of gentamicin (<500 μg/ml). Consequently, no bacteria with acquired resistance to vancomycin or gentamicin were isolated in this study.

The oral microbiota is not static; it has been shown to change in composition over time, in response to whether the individual has teeth or synthetic materials, with age, hormone status, diet, health and environment (248). This study has shown that the prevalence of bacteria resistant to three antibiotics (amoxycillin, erythromycin and tetracycline) was not significantly different within a healthy population of different origin and different background who had not taken any antibiotics for 3 months prior to sampling. Therefore, the proportion of antibiotic-resistant bacteria in the oral cavity of a healthy individual appears to be stable. However, the prevalence is likely to change when an individual takes antibiotics, which have been shown to have an

effect on the endogenous microbiota increasing both the number of resistant isolates and the MIC of the already resistant ones (59, 79, 120).

In the last few decades we have witnessed an increase in the resistance of oral viridans group streptococci to β-lactams (5, 16, 74, 126). In 1986 Koh *et al.* (140) showed that in a healthy population of 20 individuals amoxycillin-resistant streptococci comprised a mean of 0.85% of the cultivable microbiota. In this study a mean of 0.53% of streptococci were resistant to this antibiotic. Resistance of streptococci to β-lactams is still very low in the oral cavity of healthy individuals and the increase of resistance has subsequently been reported only in clinical isolates or in neutropenic cancer patients (5, 16, 74) and might be due to the consumption of antibiotics leading to an increase in the prevalence of antibiotic-resistant bacteria (59, 79, 120). A strong association was observed between penicillin and erythromycin resistance in different *Streptococcus* spp. (74, 234). In this study, 54% of amoxycillin-resistant streptococci were also resistant to erythromycin, but no linkage has been identified yet between the genes conferring resistance to β-lactam antibiotics and erythromycin.

Conversely, the mean percentage of oral bacteria resistant to amoxycillin with respect to the total oral count was quite high (4.1%) compared with that reported in other studies (85, 150, 271). However, two of these studies looked at the prevalence of β-lactamase-producing bacteria in the oral cavity of children (with a mean value of 2%) (271), and adults (mean value 1.07% of the total anaerobic count) (150) and the third study used a higher concentration of amoxycillin (2 μg/ml) and found a mean value of 0.5% (85). Therefore, it is difficult to make a direct comparison. In this project no investigation was made of the mechanism of resistance to amoxycillin or the presence of β-lactamase-producing bacteria. It is believed that one-third of β-lactam antibiotic-resistant bacteria in the oral cavity produce β-lactamases (116); if the same applies to this study, it would mean that the mean percentage of oral bacteria resistant to amoxycillin through the production of β-lactamase would be around 1.4%, which is similar to that found in previous studies (150, 271).

All of the 60 individuals harboured tetracycline-resistant bacteria in their oral microbiota. The proportion of microorganisms resistant to tetracycline was slightly

lower than reported previously (143), although Lacroix *et al.* used a lower break-point value for tetracycline (4 μg/ml). On the other hand, the values were slightly higher than those reported in the studies of Hawley *et al.* (113) and Olsvik *et al.* (209, 210, 211). However, two of these studies used a higher concentration of tetracycline to screen for resistant oral bacteria 25 μg/ml (113) and 10 μg/ml (221), whereas the other one used a lower concentration of tetracycline (1 μg/ml) (210, 211). These results are difficult to compare since these authors did not use the standard break point concentration of tetracycline. The other main difference between these studies is that, in the present study, the samples were taken from healthy individual whereas previously the samples were obtained from patients with periodontitis. Although the patients with periodontitis had not taken antibiotics 6 months prior to sampling, their condition and past history might have had an effect on the mean values of tetracycline-resistant bacteria; it is well known that antibiotic consumption induces the emergence of resistant microorganisms (209, 230), and decreases the susceptibility to the antibiotic administered (59) and that the return to a fully susceptible population is not guaranteed (81).

Great differences have been documented between different European countries in the use of systemic antibiotics (224), which was shown to have an impact on the level of resistance in the subgingival microbiota of adult patients with periodontitis (210, 211, 307) therefore this might also have an effect on the mean percentage of the resistant bacteria in the oral cavity found in different studies.

In most studies *Streptococcus* spp. appeared to be the most common of the tetracycline-resistant organisms isolated from the oral cavity; in the present study, they represented 54% of the tetracycline-resistant isolates. Many oral streptococci are naturally competent (112); this may explain why they are more frequently tetracycline-resistant than any other species. Tetracycline resistance has also been commonly found on conjugative transposons in these species and these elements are highly promiscuous (255).

As noted previously, Gram-negative bacteria (mostly Gram-negative bacilli) and anaerobes are naturally resistant to macrolides (91) while *Neisseria* spp. and *Haemophilus* spp. can be resistant to this antibiotic through the active efflux of the

drug by, *mtr* (334) and *acrAB* (257) pumps respectively, which are not specific to macrolides. Most of the Gram-negative bacteria isolated in this study were identified as *Neisseria* spp. on the basis of their atmospheric requirement, their Gram-stain (Gram-negative diplococci) and the oxidase test (oxidase positive) and some of them had a higher MIC than those normally associated with the presence of a *mtr* efflux pump (110, 187) or a chromosomal *mtr* mutations (67, 194) indicating that these organisms are likely to have acquired erythromycin resistance genes (246, 334).

The proportion of streptococci resistant to macrolides has increased for the last two decades and more particularly in *S. pyogenes* and *S. pneumoniae*, two pathogens of the upper respiratory tract (60, 83, 98, 262, 317). Erythromycin-resistant Gram-positive bacteria were the second most common resistant bacteria found in the oral cavity (with a mean average of 6.3% of the total viable count) and 34.8% of the erythromycin-resistant Gram-positive bacteria were also resistant to tetracycline. These oral bacteria can act as a reservoir for erythromycin resistance genes and they can transfer their erythromycin and tetracycline resistance genes to other more pathogenic bacteria such as *S. pyogenes* and *S. pneumoniae* (9, 218). In fact, there has been an increase in antimicrobial resistance in viridans streptococci in the last decade and particularly to β-lactam antibiotics, tetracyclines and macrolides (74, 294). In the next chapters, the results of studies on the mechanisms of resistance to tetracycline and erythromycin in the oral microbiota will be presented.

Chapter 4

Prevalence of Tetracycline Resistance Genes in Oral Bacteria

4.1 Abstract

Tetracycline is a broad-spectrum antibiotic used in humans, animals and aquaculture; therefore many bacteria from different ecosystems are exposed to this antibiotic. In order to determine the genetic basis for resistance to tetracycline in oral bacteria, saliva and dental plaque samples were obtained from two batches of 20 healthy adults who had not taken antibiotics during the previous three months. The samples were screened for the presence of bacteria resistant to tetracycline and the tetracycline resistance genes in these isolates were identified using multiplex PCR and DNA sequencing. In this group of 40 individuals a mean of 9.2% of the cultivable microbiota was resistant to 8 μg/ml of tetracycline. Most of the isolates carried tetracycline resistance genes encoding a ribosomal protection protein (RPP). The most common tetracycline resistance genes identified were *tet*(M), *tet*(W), *tet*(O) and *tet*(Q).

4.2 Introduction

Tetracycline is a broad-spectrum antibiotic, which is used in the treatment of bacterial infections in humans and animals, as well as for protozoal infections in humans; it is also used as a growth promoter in animals and in aquaculture and as an immunosuppressor in humans (45, 248). It is commonly used in dental practice as a prophylactic agent and for the treatment of oral infections (86). Low level of tetracycline is prescribed to patients with localized juvenile periodontitis since it inhibits the collagenase activity of neutrophils and thus tissue breakdown (115) The wide use of tetracycline has had the effect of exposing commensal as well as pathogenic bacteria from different ecosystems to the drug resulting in a major increase in tetracycline-resistant bacteria since this antibiotic was first used in the 1950s (45). Tetracycline acts by binding to the bacterial 30S ribosomal subunit

preventing any further protein synthesis (248). Resistance is commonly mediated by efflux of the drug or ribosomal protection; however, there are also two examples of a gene encoding a tetracycline inactivation system (72, 275). So far nine classes of genes encoding RPP have been described (Table 1.6), the most common of which is *tet*(M) (45, 248) that is commonly contained within conjugative transposons, which have an extraordinarily broad host range (45, 237, 274). The *tet*(Q) gene is also common and contained within a conjugative transposon (151), and there is also some evidence that *tet*(W) and *tet*(32) are contained within conjugative chromosomal elements although these are not yet well characterised (21, 180, 264).

Eighteen genes encoding tetracycline efflux pumps have also been described (Table 1.6). They are found in both Gram-positive and Gram-negative species. The efflux genes from Gram-negative organisms are widely distributed and are associated with large plasmids, most of which are conjugative (45). Efflux genes (*tet*(K) and *tet*(L)) from Gram-positive organisms are usually found on small transmissible plasmids (18). A novel *tet* gene *tet*(34) has recently been described from a *Vibrio* sp. (206) (see chapter 1.2.1.2.3.4).

In the oral cavity, *tet*(M) has been found in many different bacterial genera (143), *tet*(O) and *tet*(Q) were isolated in Gram-positive and Gram-negative oral species respectively (210, 211) and several efflux genes were isolated from Gram-negative oral species (248). However, the prevalence of the newly-discovered tetracycline resistance genes in the oral cavity has not been investigated. Furthermore, the presence of the different tetracycline resistance genes in healthy adults has not been investigated. Therefore, the aim of this work was to investigate the prevalence of tetracycline resistance genes in the oral microbiota, which contains many different genera and species of bacteria thereby providing ample opportunity for genetic exchange (111, 189). Oral bacteria also have the opportunity to come into contact with bacteria from other body sites (189). In this study, members of the tetracycline-resistant oral microbiota were screened for the presence of 12 different tetracycline resistance genes. It was found, for the first time, that *tet*(W), like *tet*(M), is common in oral bacteria and that *tet*(O), *tet*(Q), *tet*(A) and *tet*(S) are present in some oral species.

4.3 Materials and Methods

Multiplex PCR conditions. Bacterial DNA template was prepared using Puregene (Flowgen, Gentra System). The PCR reaction mixture (total 50 µl) included 2 µl template DNA in a final concentration of 0.1 to 1 µg, 1 x PCR buffer, 2.5 U DNA Taq polymerase (Bioline), 300 µM of each of the deoxynucleotides dATP, dCTP, dGTP, and dTTP and dH_2O. The primers and $MgCl_2$ concentrations were optimised for each multiplexed primer group according to the method described by Ng *et al.* (206). Group I contained primers for *tet*(K) (1.25 µM), *tet*(M) (0.5 µM), *tet*(O) (1.25 µM) and *tet*(S) (0.5 µM) each (3.0 mM MgCl2). Group II contained primers for *tet*(B) (0.25 µM), *tet*(C) (0.25 µM) and *tet*(D) (0.2 µM) each (4.0 mM $MgCl_2$). Individual PCR reactions were performed for *tet*(A), *tet*(Q), *tet*(W), *tet*(L) and *tet*(T); primers and PCR conditions for *tet*(W) and *tet*(T) were as described by Aminov *et al.* (7) (Table 4.1). Concerning *tet*(A), *tet*(Q), *tet*(L), primers and PCR conditions were as described by Ng *et al.* (195) (Table 4.1).

Table 4.1: Description of the primers used to detect each of the tetracycline resistance genes

	tet genes	Amplicon size (bp)	Annealing temperature	PCR conditions (Ref)
Group 1 Multiplex PCR	*tet*(B)	659	55°C	7
	tet(D)	787	55°C	7
	tet(C)	418	55°C	7
Group 2 Multiplex PCR	*tet*(K)	169	55°C	195
	tet(M)	406	55°C	195
	tet(O)	515	55°C	195
	tet(S)	667	55°C	195
Individual PCR	*tet*(A)	210	55°C	195
Individual PCR	*tet*(L)	267	55°C	195
Individual PCR	*tet*(Q)	904	55°C	195
Individual PCR	*tet*(T)	169	46°C	7
Individual PCR	*tet*(W)	168	64°C	7

A multiplex PCR was used for Group 1 and 2 and an individual PCR for *tet*(A), *tet*(L), *tet*(Q), *tet*(T) and *tet*(W).

Some of the PCR results were confirmed, where required, by DNA sequencing (see chapter 2.7).

4.4 Results

In the first Batch of 20 samples, a mean of 11% of the cultivable microbiota was resistant to 8 μg/ml of tetracycline, compared with a mean of 7.8% in Batch 2 (Table 4.2). The difference in mean proportions between the two groups was not statistically significant ($P > 0.05$). From the 20 samples processed, a representative number of 105 tetracycline-resistant bacteria were isolated for further study from Batch 1 and 104 isolates from Batch 2. Most of the isolates carried tetracycline resistance genes

encoding a RPP. The most common tetracycline resistance genes identified were *tet*(M), *tet*(W), *tet*(O) and *tet*(Q) (they represented 79%, 21%, 10.5% and 9.5% respectively of all the isolates) in Batch 1, whereas in Batch 2, *tet*(M), *tet*(O), *tet*(W) were the most common tetracycline resistance genes isolated (they constituted 78%, 9.6% and 9.3% respectively of all the isolates). No *tet*(Q) gene was identified from Batch 2. Tetracycline resistance genes encoding an efflux protein were found in only 5.7% of all the tetracycline-resistant isolates from the two batches (Table 4.2).

Table 4.2: Distribution of the tetracycline resistance genes among cultivable oral bacteria

Tetracycline-resistance genes	Batch 1 and 2 (n=209)
% of flora resistant to tetracycline	9.2% (0.34-38.9%)[a]
SD	9.5
% of isolates containing *tet*(M)	78% (164 isolates)
% of tetracycline-resistant microbiota containing *tet*(M)	77% (0-100%) [a]
SD [b]	26.1
% of isolates containing *tet*(W)	15.3% (32 isolates)
% of tetracycline-resistant microbiota containing *tet*(W)	9.8% (0-93%) [a]
SD [b]	19.6
% of isolates containing *tet*(O)	10% (20 isolates)
% of tetracycline-resistant microbiota containing *tet*(O)	9.8% (0-100%) [a]
SD [b]	19.6
% of isolates containing *tet*(Q)	5% (11 isolates)
% of tetracycline-resistant microbiota containing *tet*(Q)	4% (0-50%) [a]
SD [b]	10.9
% of isolates containing *tet*(A)	2% (4 isolates)
% of isolates containing *tet*(K)	1.4% (3 isolates)
% of isolates containing *tet*(L)	1.4% (3 isolates)
% of isolates containing *tet*(S)	1% (2 isolates)
% of isolates containing *tet*(B)	1% (2 isolates)

[a] The percentages in brackets represent the range of values found in the 40 samples studied.

[b] The standard deviation (SD) gives an indication on how spread the data are.

The difference in mean proportions, between the two batches, of the three most common tetracycline resistance genes identified (*tet*(M), *tet*(W) and *tet*(O), excluding *tet*(Q), which was only found in Batch 1) were compared using the Kruskal Wallis test. The difference in the mean proportion of the tetracycline resistance genes for each batch was considered significant when the *P* value was <0.05. No significant differences were found in the prevalence of the three tetracycline resistance genes in the oral cavity in the two batches (Table 4.3) therefore Batch 3 was not further investigated. The data from Batch 1 and 2 were combined and analysed together.

Table 4.3: Comparison of the mean proportions, between the two patient groups, of the three most common tetracycline resistance genes identified using the Kruskal-Wallis test

Tetracycline-resistance gene	*P* value[a]
tet(M)	0.27
tet(W)	0.62
tet(O)	0.46

[a] The difference in the mean proportion of the tetracycline resistance genes for each patient group was considered significant when the *P* value was < 0.05

tet(M) was the most common tetracycline resistance gene isolated (Table 4.2); it was isolated from 39 samples. In this group of 40 individuals, between 0% and 100% (mean = 77 %, SD = 26.1) of tetracycline-resistant bacteria harboured the *tet*(M) gene. 15% (n=25) of isolates carrying *tet*(M) had one or two other tetracycline resistance genes. However, this did not have any effect on the MIC of tetracycline since the isolates with one tetracycline resistance gene and the isolates with at least two had the same MIC_{50} and MIC_{90}, which were 16 μg/ml and 32 μg/ml respectively.

tet(W) was the second most common tetracycline resistance gene identified. Between 0.0% and 93% (mean = 9.8 %, SD = 19.6) of tetracycline-resistant bacteria harboured the gene (Table 4.2). The isolates carrying this gene were identified by

16S rRNA gene sequencing as *Veillonella, Prevotella, Streptococcus, Staphylococcus, Rothia, Lactobacillus* or *Neisseria* spp. (Table 4.4). Out of all the isolates containing *tet*(W), 34% (11 isolates out of 34) contained one or two other tetracycline resistance genes *tet*(M) (in 9 isolates), *tet*(O) (one isolate) and *tet*(L) (one isolate) (Table 4.4).

Table 4.4: Tetracycline resistance genes detected for the first time in oral species

Genus	*tet* genes detected for the first time in a particular genus	Number of times it was found in each genus	Other *tet* genes found in the same isolate (and number of isolates with at least two *tet* genes)[a]
Rothia	*tet*(W)	8	*tet*(M) (1)
Actinomyces	*tet*(W)	6	*tet*(M) (1) *tet*(L) (1)
Streptococcus	*tet*(W)	6	*tet*(M) (2)
Neisseria	*tet*(W)	5	*tet*(M) (1) *tet*(O) (1)
Lactobacillus	*tet*(W)	3	-
Veillonella	*tet*(W)	2	*tet*(M) + *tet*(S) (1) *tet*(M) (1)
Bacillus	*tet*(W)	1	-
Staphylococcus	*tet*(W)	1	-
Veillonella	*tet*(S)	2	*tet*(M) + *tet*(A) (1) *tet*(M) + *tet*(W) (1)
Neisseria	*tet*(O)	1	*tet*(W) (1)
Neisseria	*tet*(Q)	1	*tet*(M) (1)
Veillonella	*tet*(A)	1	*tet*(M) + *tet*(S) (1)

[a] The last column gives an overview of the isolates containing more than one tetracycline resistance gene. For instance, in the first row, out of 8 *Rothia* spp. containing *tet*(W), one isolate also carried *tet*(M)

tet = tetracycline

The *tet*(O) and *tet*(Q) genes were detected for the first time in *Neisseria* spp.

4.5 Discussion

Resistance to tetracycline through the carriage of *tet*(M) was found to be widespread among the 40 healthy individuals studied (77% of the tetracycline-resistant microbiota contained *tet*(M)) who had not taken any antibiotics in the previous 3 months. Tet M has been considered to be the most widespread tetracycline determinant in different environments and has been found in a variety of Gram-positive and Gram-negative bacteria (248). The wide distribution of *tet*(M) probably reflects the fact that it is contained within broad host range conjugative transposons which have previously been found to be common in oral bacteria (19, 51, 237, 243).

This is the first report of the isolation of the *tet*(W) gene in bacteria colonising the human oral cavity. It was the second most common tetracycline resistance gene in the cultivable bacteria (Table 4.2). *tet*(W) was found for the first time in *Veillonella, Prevotella, Streptococcus, Staphylococcus, Rothia, Actinomyces, Lactobacillus* and *Neisseria* spp. (Table 4.4). This gene was originally identified in the bovine rumen anaerobe *Butyrivibrio fibrisolvens* and subsequently in human faecal anaerobes, pigs and recently in a facultative anaerobe *Arcanobacterium pyogenes* (17, 21, 264). In *B. fibrisolvens tet*(W) is contained within a large conjugative transposon, Tn*B1230* (17) which is capable of high frequency conjugative transfer among *B. fibrisolvens* species. In *A. pyogenes tet*(W) was associated with a *mob* gene, not found in Tn*B1230,* and was capable of conjugative transfer at low frequency (21), indicating that there are at least two different genetic elements responsible for the spread of this resistance gene.

Recently a new organisation of *tet*(W) gene was revealed in *Megasphaera elsdenii*, a commensal anaerobic swine strain; the tetracycline resistance determinant of this strain consisted of mosaic genes formed by recombination of *tet*(O) and *tet*(W) class genes (279), this mosaic organisation was subsequently found in different strains isolated from the faeces of organically raised swine (278). Characterisation of the genetic elements carrying *tet*(W) in the oral cavity will be described in Chapter 7.

The *tet*(O) gene was the third most common tetracycline resistance gene isolated in this study (Table 4.2). This gene has been found in different species (45) but in the oral/respiratory tract it has been isolated only in Gram-positive species (*Lactobacillus, Enterococcus, Staphylococcus and Streptococcus*) (248). In this study, *tet*(O) was found for the first time in one *Neisseria* sp. (Table 4.4). The *tet*(O) gene was initially identified on R plasmids harboured by the Gram-negative *Campylobacter coli* (196) and was subsequently found on the chromosome of *Streptococcus* spp. where it is associated with conjugative elements (101). Because of a G+C content (40%) higher than that of *Campylobacter coli* chromosomal and plasmid DNAs (31 to 33% respectively) (339), and because this tetracycline resistance gene exhibits high sequence identity with *tet*(M) from *Streptococcus* sp. (273) it is thought that *tet*(O), like *tet*(M), originated in a Gram-positive bacterium.

tet(Q) has been reported to be common in Gram-negative oral bacteria that are associated with periodontal tissue destruction (142, 211). However, the current study has shown for the first time that *tet*(Q) is present in a commensal *Neisseria* sp. (Table 4.4). Previous work has shown that the *tet*(Q) gene is frequently associated with conjugative transposons in the *Bacteroides* group (151, 199, 269). These elements were found to have a broad host range and may be responsible for the wide dissemination of the *tet*(Q) gene in oral bacteria; this has not been investigated in this project but would be an obvious area of research for the future.

Out of the 209 tetracycline-resistant isolates, the *tet*(S) gene was found only in two different Gram-negative anaerobes (*Veillonella* sp.) from two different samples (Table 4.4), it is the first time that *tet*(S) has been isolated in this genus. *tet*(S) was first detected in *Listeria monocytogenes* BM4210 where it is carried by self-transferable plasmids (43). It has also been found on the chromosome of *Enterococcus faecalis* where it is associated with a 40-kb fragment that can be mobilised from chromosome to chromosome only in the presence of the conjugative plasmid pIP825 (42, 93). Recently, *tet*(S) was shown to be contained within a functional Tn*916*-like element, in the same relative position as *tet*(M) (144).

tet(A), *tet*(S) and *tet*(W) were isolated from *Veillonella* spp. for the first time (Table 4.4). Previously the only tetracycline resistance genes isolated from this organism were *tet*(L), *tet*(M) and *tet*(Q) (45). This shows that *Veillonella* spp. may be an important reservoir of different tetracycline resistance genes.

The carriage of more than one tetracycline resistance gene was commonly found. However, as indicated earlier, this did not have any effect on the MIC of the organism indicating that a need for an increased level of tetracycline resistance is not the selective pressure for this phenomenon. Rather this could be because some of the tetracycline resistance genes are contained within conjugative transposons (i.e. *tet*(M), *tet(*Q), *tet*(W) and *tet*(S)). The possession of one conjugative transposon is not a barrier to that same cell being able to receive other related or unrelated conjugative transposons (45, 189). It has also been shown that some bacteria are naturally competent (*Neisseria*, *Haemophilus*, *Streptococcus* spp.), which could help further dissemination of the tetracycline resistance genes (248).

In conclusion, this study has shown that bacteria in the oral cavity contain a variety of tetracycline resistance genes, indicating that oral bacteria have access to and/or are a reservoir of resistance genes, and that *tet*(O), *tet*(W), *tet*(S) and *tet*(Q) genes have spread across a broad spectrum of bacterial species. Next, it is important to determine the genetic supports for some of these resistance genes in order to understand how they disseminate.

Chapter 5

Characterisation of the Genetic Basis for Erythromycin Resistance in Oral Bacteria

5.1 Abstract

There has been an increased rate of resistance to erythromycin in clinical as well as commensal isolates, which correlates with an increased use of this class of antibiotics during the last two decades. Resistance to macrolides is commonly mediated by methylases (encoded by *erm* genes), by efflux of the drug, by production of inactivating enzymes or, to a lesser extent, by mutation in the ribosomal binding site. The aim of this study was to determine the molecular basis for erythromycin resistance in oral bacteria. Twenty saliva and plaque samples from healthy adults were screened for the presence of erythromycin-resistant bacteria and 122 (37 Gram-positive and 85 Gram-negative) resistant strains were isolated. All of the resistant isolates were screened for possession of the most common erythromycin resistance genes by PCR. Forty-two isolates (mostly Gram-positive) contained at least one erythromycin resistance gene, whereas eighty Gram-negative isolates did not contain any detectable resistance genes. The *mef* gene was the most commonly found, followed by *erm*(B).

5.2 Introduction

The macrolide erythromycin or its derivatives are commonly used in poultry, livestock and human clinical practice to prevent or treat infections due to Gram-positive bacteria. In humans, macrolides are used for the treatment of respiratory tract infections or as an alternative in patients who are allergic to penicillin.

Macrolides bind to the 50S ribosomal subunit stimulating dissociation of the peptidyl-tRNA from the ribosome during elongation, resulting in peptide chain

termination and reversible stoppage of protein synthesis. This binding site overlaps with the binding site of the newer macrolides, ketolides and the structurally unrelated lincosamides and streptograminB antibiotics (148).

There are three main mechanisms conferring macrolide resistance. Acquisition of a ribosomal methylase (erm) is the most common (Table 1.5). Methylation causes a conformational change in the prokaryotic ribosome and confers resistance to macrolides, lincosamides and streptogramins, giving the MLS phenotype (247). Efflux proteins give rise to resistance by pumping the antibiotic out of the cell keeping the intracellular concentration of the antibiotic low and the ribosomes free from the action of the antibiotic. There are two types of efflux pump: *msr*A/B is most commonly found in *Staphylococcus* spp. and the *mef* gene is most commonly found in *Streptococcus* spp. (148) but can also be detected in *Neisseria* spp. (247). The *mef* gene confers resistance only to macrolides, giving the isolate the M phenotype whereas the *msrA* gene confers resistance to macrolides and streptogramins, giving the isolate the MS phenotype (148). Resistance can also be conferred by the production of enzymes, which inactivate erythromycin only, esterase (*ereA*, *ereB*) and phosphotransferases (*mph*); these enzymes have been found in highly macrolide-resistant *Enterobacter* spp. (8, 91).

A fourth mechanism of resistance, a base substitution in the 23S rRNA or in the ribosomal proteins has emerged in clinical isolates since the 1990s (see chapter 1.2.1.2.3.3).

A healthy oral microbiota consists mostly of *Streptococcus* spp. including viridans group streptococci (VGS), which have already been studied for the presence of erythromycin resistance genes and VGS is likely to be an important reservoir for the dissemination of these genes to more pathogenic species (9, 10, 218, 265). *Neisseria* and *Haemophilus* spp. can harbour an efflux pump (*mtr* and *acrAB* respectively); these pumps reduce the outer membrane permeability of the isolate to dyes and detergents and increase the resistance to multiple antimicrobial agents such as macrolides, however they confer only low level resistance to this class of antibiotics (187, 334). Moreover, some Gram-negative bacteria are intrinsically resistant to

macrolides due to the low permeability of their outer membrane to these hydrophobic compounds (91).

5.3 Materials and Methods

PCR reactions. The resistant isolates were tested for the presence of erythromycin resistance genes using either individual PCR assay as for *mph*(A) (284) or a multiplex PCR. Group I and II multiplex PCR included *erm*(B) and *erm*(C), and *erm*(A), *erm*(F), *msr*(A) respectively using the same conditions as in Nawaz *et al.* (193). Group III multiplex PCR consisted of *ere*(A), *ere*(B) and *mef*(A) as stated in Sutcliffe *et al.* (284).

All of the PCR reactions were performed on DNA isolated using the Puregen Kit (Flowgen, Gentra System). Some of the PCR results were confirmed, when required, by DNA sequencing (see chapter 2.7).

Antibiotic susceptibility. The different macrolide resistance phenotypes were identified using discs containing erythromycin (30 μg) or clindamycin (10 μg) as described by Seppäla *et al.* (265). Each isolate was resuspended in tryptone soya broth (Sigma) to a density of 0.5 McFarland units and was also tested for its susceptibility to azithromycin (100 μg) and spiramycin (100 μg) disks (Oxoid) on Mueller-Hinton agar (Oxoid) with 5% defibinated horse blood (E & O laboratories, Bonnybridge, Scotland). All of the Gram-negative isolates were tested for their susceptibility to crystal violet (Sigma) and Triton X-100 (Sigma) by the agar plating method described by Morse *et al.* (187).

5.4 Results

In Batch 1, 20 % of the cultivable microbiota from the 20 samples was found to be erythromycin-resistant. From the 20 samples processed, a representative number of 122 erythromycin-resistant bacteria were selected for further study. Most (70%) of the isolates were Gram-negative and did not contain previously identified

erythromycin resistance genes. Most of the 42 isolates found to carry at least one erythromycin resistance gene were Gram-positives from the *Streptococcus* genus. Therefore, the mean percentage of erythromycin-resistant isolates with an identified erythromycin resistance gene was only 7%.

Most of the Gram-negative bacteria with no identified erythromycin resistance gene had a very low MIC (ranging from 1 μg/ml to 8 μg/ml) and 26.5% of all the Gram-negative isolates were resistant to an elevated concentration of both a dye, crystal violet, and a detergent, Triton X-100, thus according to Shafer *et al.* these isolates are likely to be resistant to erythromycin due to the presence of a non-specific *mtr* efflux pump (187, 334).

The antimicrobial susceptibility patterns for erythromycin, clindamycin, azithromycin and spiramycin were checked in the 42 isolates containing an identified erythromycin resistance gene using the disc method. These isolates exhibited two different phenotypes: 67% had the M phenotype; they were fully resistant to macrolides (erythromycin, azithromycin and spiramycin) and displayed variable zones of inhibition around the clindamycin disc, and 33% had the MLS phenotype; they were fully resistant to macrolides and lincosamide (clindamycin) (Table 5.1).

Table 5.1: Distribution of erythromycin resistance genes in the oral microbiota

Class of resistance gene to macrolides	Nb of isolates	Genus[a]	MIC µg/ml[b] Ery[c]	Erythromycin resistance gene(s)	Resistance to ery, clin, azm, spy disks
Methylase	11	*Streptococcus*	64	*erm*(B)	Fully resistant
	1	*Streptococcus*	> 128	*mef* + *erm*(B)	Fully resistant
	1	*Veillonella*	32	*erm*(B)	Fully resistant
	1	*Prevotella*	4	*erm*(F)	Fully resistant
Efflux	21	*Streptococcus*	4	*mef*	Variable zones of inhibition
	1	*Streptococcus*	> 128	*mef* + *erm*(B)	Fully resistant
	3	*Neisseria*	4-8	*mef*	Variable zones of inhibition
	2	*Lactobacillus*	2-8	*mef*	Variable zones of inhibition
	1	*Staphylococcus*	> 128	*msr*(A)	Variable zones of inhibition

[a] The MIC breakpoint to erythromycin for *Streptococcus*, *Staphylococcus*, *Neisseria* spp. is < 0.5 µg/ml, and for anaerobes is < 2 µg/ml according to the British Society for Antimicrobial Chemotherapy (http://www.bsac.org.uk/).

[b] The MIC_{50} and MIC_{90} of all the isolates with a methylase gene was 64 µg/ml and 128 µg/ml respectively and the MIC_{50} and MIC_{90} of all the isolates with an efflux gene was 4 and 16 µg/ml respectively.

Nb = number, ery = erythromycin, clin = clindamycin, azm = azithromycin, spy = spiramycin.

Of 28 M-phenotype isolates, 27 were positive with primers specific for the *mef* gene and one was positive with primers specific for the *msr*(A) gene. Of the 14 MLS-phenotype isolates, 13 were positive with primers specific for the *erm*(B) gene and one was positive with primers for the *erm*(F) gene (Table 5.1). One isolate with the MLS phenotype carried both the *erm*(B) and *mef* genes.

The MIC_{50} and MIC_{90} of all the isolates with a methylase gene were 64 μg/ml and 128 μg/ml respectively and the MIC_{50} and MIC_{90} of all the isolates with an efflux gene were 4 μg/ml and 16 μg/ml respectively.

The susceptibility of the isolates to amoxycillin and tetracycline was also determined. Only 6 isolates were also resistant to amoxycillin with a low MIC (1 μg/ml), except for one that had a MIC of 32 μg/ml. Five of these isolates carried the *mef* gene and one isolate with the high MIC for amoxycillin carried the *msrA* gene although β-lactam antibiotics are not known to be substrates for these efflux pumps (150). Out of the 42 isolates with an identified erythromycin resistance gene, 12 (28%) were also resistant to tetracycline; these last results will be further discussed in Chapter 6.

The 42 isolates were identified to the genus level by biochemical tests and partial 16S rRNA sequencing (Table 5.1). Most of them belonged to the *Streptococcus* genus (34 out of 42, 81%) and had either the efflux or methylase genes.

5.5 Discussion

There has been an increased rate of resistance to erythromycin in clinical as well as commensal isolates, which correlates with an increased use of this class of antibiotics during the last two decades (134, 165, 234, 285, 333). The current study found that 7% of the oral bacteria from samples obtained from 20 individuals carried an erythromycin resistance gene. Most of the erythromycin-resistant isolates with an identified resistance gene were streptococci and the most common resistance gene was *mef* followed by *erm*(B). These results agree with previous studies which have looked at the prevalence of the erythromycin resistance genes in this genus and found

that viridans streptococci from pharyngeal samples are a reservoir of *erm*(B) and *mef* genes (9, 218). It was not surprising to find that most of the erythromycin-resistant isolates were streptococci since viridans streptococci are important constituents of the indigenous microbiota of the upper respiratory tract of healthy humans (14).

As noted in previous studies (9, 60, 83, 102, 127, 165, 317), there was a correlation between the antibiotic resistance phenotype and genotype for each isolate. The isolates with a methylase gene were fully resistant to the macrolides (erythromycin, azithromycin and spiramycin) and to the lincosamide class of antibiotics (clindamycin) whereas the isolates with a *mef* gene displayed varying zones of inhibition around the antibiotic discs (Table 5.1). The MIC_{50} and MIC_{90} were higher in the isolates with a methylase gene than those with a *mef* gene as already noted in *S. pneumoniae* (97), therefore, the isolates with an *erm* gene were more resistant to erythromycin than the isolates with a *mef* gene. One isolate with both genes had the same phenotype as the isolates with the *erm*(B) gene only; the identification of both genes in the same isolate and the expression as MLS phenotype has already been described in *Streptococcus pneumoniae* and *Streptococcus pyogenes* (83, 165, 175, 218).

Perez-Trallero *et al.* showed that 94.4% of a total of 197 subjects carried erythromycin-resistant commensal streptococci in their pharynx and that there were no statistical differences in the number, the species or the resistance phenotypes of erythromycin-resistant streptococci found in healthy subjects or subjects with pharyngitis (218). Moreover, half of the erythromycin-resistant streptococci had the M phenotype and the other half had the MLS phenotype. In this study, a high percentage of individuals (80% out of a total of 60 subjects) also carried erythromycin-resistant streptococci in their oral cavity. However, the distribution of the phenotypes was different since 67% had the M phenotype and 33% had the MLS phenotype. This difference in the prevalence of the methylase genes and the efflux genes in some clinical and commensal streptococcal isolates has been observed in different studies (83, 97, 175, 191, 285). It appears that the resistance pattern to macrolides in *Streptococcus* spp. depends on the geographical region and more particularly on the macrolide used in this particular region; a positive correlation was found between azithromycin use and an increase in macrolide resistance (335),

however no study has ever shown the link between the use of a macrolide and the prevalence of a particular erythromycin resistance gene.

One of the reasons for the dissemination of the *mef* gene in VGS is the presence of this gene on mobile elements. Recently the *mef*(A) gene was shown to be part of a 7.2-kb non-conjugative transposon Tn*1207.1* (260) that was subsequently reported to be part of a larger conjugative transposon, Tn*1207.3* (259). The *mef* gene was also found to be inserted into a Tn*916*-like element, in a sequence homologous to *orf6* of Tn*916* (Fig 1.8) forming the new element Tn*2009* (68).

The current study showed that a methylase gene, *erm*(B), was isolated for the first time from a *Veillonella* sp. *Veillonella* spp. have already been shown to be an important reservoir for different tetracycline resistance genes (see Chapter 4). The remaining Gram-negative isolates with no identified erythromycin resistance genes that do not present a mtr phenotype are likely to be intrinsically resistant to macrolides (91) and this is supported by their low MIC to erythromycin. None of the Gram-negative isolates with a known erythromycin resistance gene had the mtr phenotype.

No *ermA* genes were detected during this study although this gene has previously been found in *Peptostreptococcus* spp., a normal member of the pharyngeal, dental and gingival microbiota, and was shown to transfer, albeit at low frequency, to a *Streptococcus pyogenes* recipient (233); it is possible that no *Peptostreptococcus* spp. were isolated in this study because the collection or transport of specimens were not optimised enough for the recovery of these Gram-positive anaerobic cocci (190). A previous study looking at the distribution of *mef* and *erm* genes in commensal streptococci resistant to erythromycin did not isolate any *erm*(A) in a total of 253 isolates (218), thus *erm*(A) is not commonly isolated from the oral cavity.

No PCR products were detected with primers for *ere*(A), *ere*(B) and *mph*, but this was not surprising since these genes are most commonly found in highly resistant *Enterobacter* spp. (8, 11, 205) and they have not been isolated from *Streptococcus* spp.

Ribosomal mutation conferring resistance to macrolides has emerged in the last few years as the sole mechanism of resistance in clinical isolates of mostly *S. pneumoniae* (289) and *S. pyogenes* (23). However, all of the erythromycin-resistant streptococci isolated in this study contained either a *mef* or *erm* gene. Ribosomal mutations might have appeared due to the selective pressure of increased levels of exposure to macrolides in recent years (289), but it is not found as the sole mechanism of resistance to macrolides in oral commensal streptococci, however there is still the possibility that it could be found in conjunction with a resistance gene, explaining why it was not detected in this study.

In this study, commensal streptococci of the oral cavity were shown to be an important reservoir for erythromycin resistance genes and may be a reservoir for the dissemination of the *erm*(B) and the *mef* genes to more pathogenic bacteria such as *S. pyogenes* and *S. pneumoniae*. In the next chapter, the transfer of antibiotic resistance genes between the oral streptococci and *Enterococcus faecalis* will be examined.

Chapter 6

Transfer of Genes Encoding Antibiotic Resistance

6.1 Abstract

Of the 42 isolates that contained an identified erythromycin resistance gene, 12 were also resistant to tetracycline and 4 out of these 12 isolates were shown to transfer both genes simultaneously to an *Enterococcus faecalis* recipient in filter-mating experiments. Hybridisation analysis showed that the transferred genes were contained within a conjugative transposon related to Tn*1545*.

6.2 Introduction

Conjugative transposition is one of the most common mechanisms of horizontal gene transfer between organisms of different species or different genera (255). The Tn*916*-Tn*1545* family of elements are the best-studied conjugative transposons; they have been found in a wide variety of Gram-positive and Gram-negative organisms (51), and they appear to have played a significant role in the dissemination of multiple antibiotic resistance among clinically important species such as streptococci (237, 267). Both transposons contain *tet*(M), identical ends and similar integrase and excisase genes (called *int/xis* respectively) (51). Tn*1545* carries two extra antibiotic resistance genes, *erm*(B) and *aphA*-3, conferring resistance to macrolides and kanamycin respectively. Although this family of conjugative transposons has a wide host-range this study is the most complete investigation of Tn*1545* elements in viridans streptococci.

6.3 Materials and Methods

Detection of erythromycin and tetracycline resistance determinants. The erythromycin-resistant isolates from Batch 1 that had an identified erythromycin resistance gene (see chapter 5) and that were also resistant to tetracycline were tested for the presence of tetracycline resistance genes using multiplex PCR for *tet*(K), *tet*(M), *tet*(O) and *tet*(S) as described in Ng *et al.* (195), or an individual PCR assay for *tet*(W) as described in Aminov *et al.* (7).

Mating experiments. Each isolate with an identified erythromycin resistance gene and which was also resistant to tetracycline was tested for its ability to simultaneously transfer both genes to *Enterococcus faecalis* JH2-2 which was resistant to rifampicin (see chapter 2.8). The experiment was repeated four times: the transconjugants were selected twice on tetracycline (8 μg/ml) and rifampicin (25 μg/ml) plates and twice on erythromycin (10 μg/ml) and rifampicin (25 μg/ml) plates. Each transconjugant was also tested for its resistance to the non-selected marker by plating it onto tetracycline or erythromycin-containing plates. The co-transferred resistance genes were identified by PCR and Southern blot for each transconjugant.

Hybridisation. Total DNA from the donors and transconjugants was digested with *Hin*dIII, run into an agarose gel and transferred to Hybond-N+ membrane by Southern blotting (see chapter 2.9). Hybridisations were performed with *tet*(M), *erm*(B), plasmid pAM120 (containing Tn*916*), plasmid pPPM70 (containing *aphA*-3) or *int/xis* probes (Appendix 1). The probes were either made from PCR products of the respective control strains (Appendix 3), which were then purified using a PCR purification kit (Sigma) or from the whole plasmids pAM120 and pPPM70 (Appendix 1). The hybridisation blots were developed and exposed to Hyperfilm ECL (Amersham) for 5 min and overnight.

6.4 Results

In the course of this study, 12 isolates were identified that, as well as being resistant to erythromycin, were also resistant to tetracycline (Table 6.1). The most common antibiotic resistance genes identified were *erm*(B) and *tet*(M) (Table 6.1). One isolate contained *msr*(A) and *tet*(W) but no transfer was detected from this strain (Table 6.1).

Four isolates out of 12 could transfer tetracycline and macrolide resistance to the *Enterococcus faecalis* JH2/2 recipient strain under the conditions used (Table 6.1). The frequencies of transfer from the 4 donors were variable according to the antibiotic used for selection. Donors E25-3 and E31-2 did not transfer at detectable frequencies when tetracycline was used as the selective antibiotic but were able to transfer at an average frequency of 3.3×10^{-9} and 1.4×10^{-7} transconjugants per donor respectively when erythromycin was used as the selective agent. However donors E37-4 and E38-2 had a higher transfer frequency when tetracycline was used as the selective antibiotic (5.15×10^{-4} and 1.5×10^{-5} transconjugants per donor respectively) whereas the frequencies of transfer were 2.6×10^{-5} and 7×10^{-8} transconjugants per donor respectively when erythromycin was used as the selective agent (Table 6.1).

All the transconjugants were resistant to both erythromycin and tetracycline and contained both *tet*(M) and *erm*(B). The four different donors belonged to the *Streptococcus* genus; the MICs of the transconjugants for the two different antibiotics were similar to those of the donors (Table 6.1).

Table 6.1: Results of the filter-mating experiments between strains resistant to both erythromycin and tetracycline as donors and *E. faecalis* JH2/2 as the recipient

Isolate	Genus	Transcon-jugant	Erm^r/ Tc^r genes from the donor and their respective MIC (μg/ml)	Erm^r/Tc^r genes from the transconjugant and their respective MIC (μg/ml)	Frequency of transfer[a] (CFU/donor cell)
E21-3	*Staph*	nd	*msr*(A)/ *tet*(W) (128 and 128)	nd	
E23-4	*Strep*	nd	*erm*(B)/ *tet*(M) (32 and 16)	nd	
E24-2	*Strep*	nd	*erm*(B)/ *tet*(M) (128 and 16)	nd	
E25-3	*Strep*	E25-3 x *E. faecalis*	*erm*(B)/ *tet*(M) (64 and 32)	*erm*(B)/ *tet*(M) (128 and 32)	$5.8\ 10^{-9}$ / $8.6\ 10^{-8}$
E26-5	*Strep*	nd	*erm*(B)/ *tet*(M) (128 and 32)	nd	
E31-2	*Strep*	E31-2 x *E. faecalis*	*erm*(B)/ *tet*(M) (4 and 32)	*erm*(B)/ *tet*(M) (128 and 64)	$1.5\ 10^{-7}$ / $1.3\ 10^{-7}$
E31-3	*Strep*	nd	*erm*(B)/ *tet*(M) (64 and 16)	nd	
E33-3	*Strep*	nd	*mef*/ *erm*(B)/ *tet*(M) (128 and 16)	nd	
E37-4	*Strep*	E37-4 x *E. faecalis*	*erm*(B)/ *tet*(M) (128 and 32)	*erm*(B)/ *tet*(M) (128 and 64)	$1.3\ 10^{-7}$ / $5.1\ 10^{-5}$ ($8.5\ 10^{-4}$ / $1.8\ 10^{-4}$)
E38-2	*Strep*	E38-2 x *E. faecalis*	*erm*(B)/ *tet*(M) (128 and 32)	*erm*(B)/ *tet*(M) (128 and 64)	$5.4\ 10^{-8}$ / $8.6\ 10^{-8}$ ($2.6\ 10^{-5}$ / $3.7\ 10^{-6}$)
E38-4a	*Veill*	nd	*erm*(B)/ *tet*(M) (32 and 8)	nd	
E38-5	*Strep*	nd	*erm*(B)/ *tet*(M) (64 and 32)	nd	

[a] Frequency of transfer found when erythromycin was used as the selective agent; in brackets are the frequencies of transfer found when tetracycline was used as the selective agent.

Highlighted in yellow are the isolates that co-transferred both resistance genes to *E. faecalis* JH2/2 recipient strain.

Erm^r = erythromycin resistance, Tc^r = tetracycline resistance, nd = not detected, *Staph* = *Staphylococcus*, *Strep* = *Streptococcus*, *Veill* = *Veillonella*.

The parents and transconjugants were subjected to a Southern blot analysis. The analysis of the blots showed at least two different Tn*1545*-type genetic elements (Fig 6.1). In one of which the *erm*(B) and *tet*(M) genes are contained on the same 4.8-kb *Hin*dIII fragment however *aphA*-3 and *erm*(B), *int/xis* and *tet*(M) are contained on a different *Hin*dIII fragment (Fig 6.1); see lanes 4 (donor E25-3), 5 (transconjugant E25-3 x *E. faecalis*), 6 (donor E31-2), 7 (transconjugant E31-2 x *E. faecalis*), 10 (donor E38-2), 11 (transconjugants E38-2 x *E. faecalis*). In the second Tn*1545*-type genetic elements, the *erm*(B) and *tet*(M) genes are not contained on the same *Hin*dIII fragment, however *int/xis* and *tet*(M) as well as *aphA*-3 and *erm*(B) are contained on the same *Hin*dIII fragment; see lanes 8 (donor E37-4) and 9 (transconjugant E37-4 x *E. faecalis*) (Fig 6.1).

Furthermore, the donor E25-3, lane 4, contains at least one copy of the Tn*1545* element (the *erm*(B), *tet*(M), *int/xis* and *aphA*-3 genes are contained within a Tn*916*-like element) however, the hybridization blots showed the presence of three bands when using *erm*(B) and *tet*(M) as probes and identical band size are also present in the pAM120 blot, this might reflect the presence of a *Hin*dIII restriction site within these two antibiotic resistance genes.

Donor E31-2, lane 6, contains only one copy of Tn*1545*, however the transconjugant E31-2 x *E. faecalis* in lane 7 received two copies of the same element; the use of *erm*(B), *tet*(M) and *int/xis* as probes resulted in the presence of two bands in each blot and identical band size are also present in the pAM120 blot. However, the hybridization with *aphA*-3 probe resulted in the presence of one band only but the band is very thick and might be due to the presence of two copies of the *aphA*-3 gene with bands of similar size juxtaposed to each other.

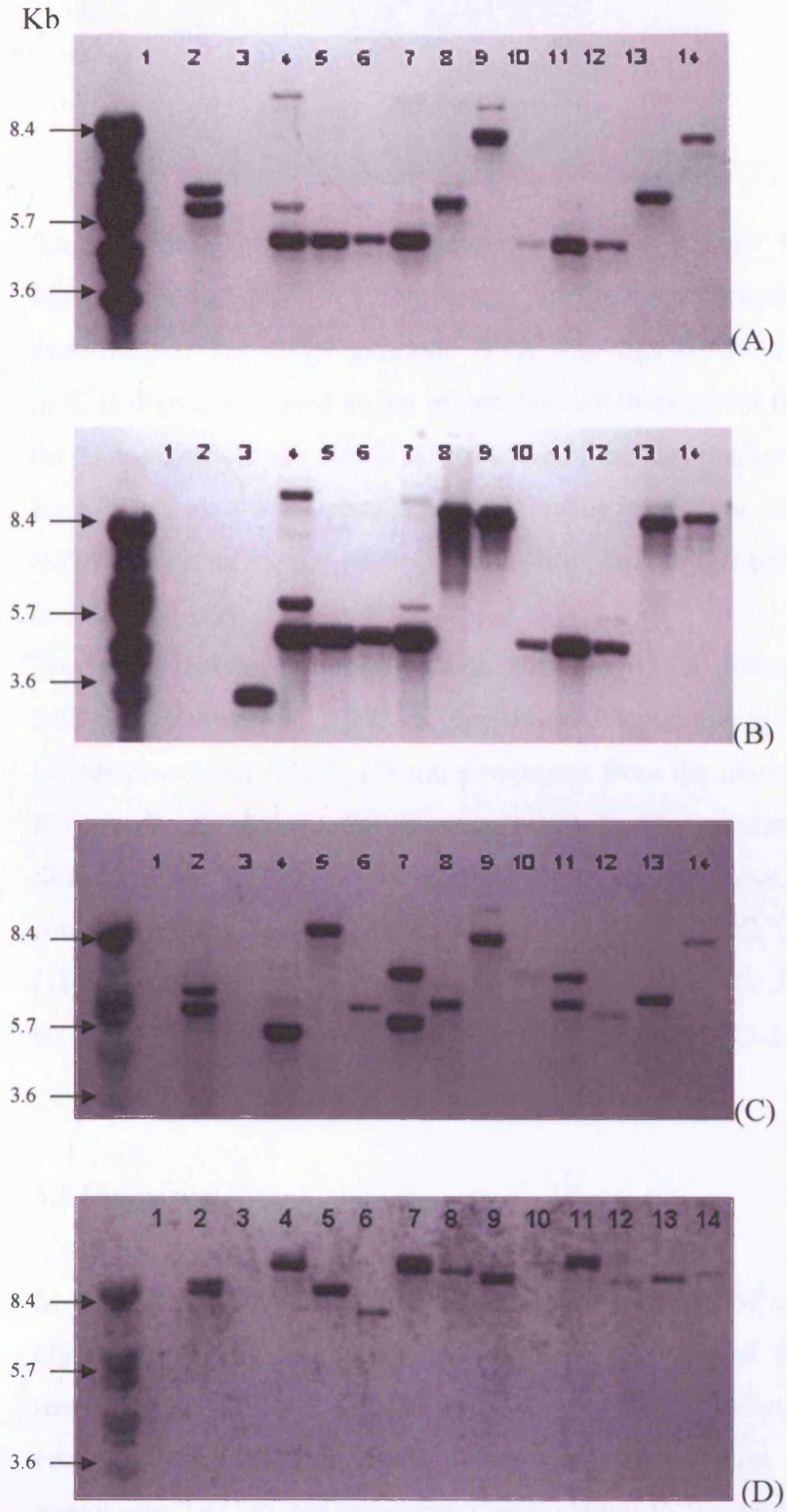
Transconjugant E37-4 x *E. faecalis* in lane 9 received one copy of the Tn*1545* element. Each blot also showed the presence of a high molecular weight band that hybridized with each probe and it is likely due to the presence of undigested DNA (Fig 6.1).

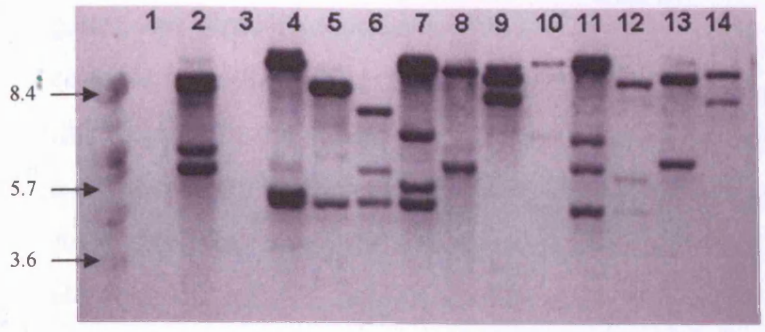
Donor E38-2, lane 10, contains *erm*(B) and *tet*(M) on the same *Hin*dIII fragment, however the band is not showing on the pAM120 blot and might be due to a low

DNA concentration during digestion and/or a short exposure time during the development of the hybridization blot. Transconjugant E38-2 x *E. faecalis* in lane 11 received two copies of the Tn*1545* element; showed by the presence of two bands for *int/xis*. The occurrence of a thick band for *erm*(B), *tet*(M) and *aphA*-3 might be due to the presence of two copies of the genes with bands of similar size juxtaposed to each others (Fig 6.1).

Donors E23-4, lane 12, E24-2, lane13, and E33-3, lane 14, did not transfer any of their resistance genes under the conditions used. Donor E23-4 contains *erm*(B) and *tet*(M) genes on the same 4.8-kb *Hin*dIII fragment however *aphA*-3 and *erm*(B), *int/xis* and *tet*(M) are contained on a different *Hin*dIII fragment whereas donors E24-2 and E33-3 contain *int/xis* and *tet*(M) on the same *Hin*dIII fragment as well as *aphA*-3 and *erm*(B) but *erm*(B) and *tet*(M) genes are not contained on the same *Hin*dIII fragment (Fig 6.1).

Figure 6.1: Analysis of the conjugative element involved in the transfer of *erm*(B) and *tet*(M) by southern blotting





(E)

Southern blot hybridisation analysis showing a linkage between *tet*(M), *erm*(B), *int/xis* and *aphA*-3 genes in the parents and the transconjugants from the filter-mating experiments. The entire genomic DNA was digested with *Hin*dIII. Lambda DNA-*Bst*E II digest was used as the molecular size marker and the numbers on the left of the blots represent the size in KB of some of the size markers.

Each panel shows the results of probing the same blot with a different probe (A) *tet*(M), (B) *erm*(B), (C) *int/xis*, region from Tn*916,* (D) pPPM70 containing *aphA*-3 and (E) pAM120.

The lanes contain genomic DNA as indicated (1) *E. faecalis* JH2/2, (2) *B. subtilis* BS34A (see Appendix 1), (3) S. *pyogenes* AC1 containing *erm*(B) (see Appendix 1), (4) *Streptococcus* E25-3, (5) transconjugant from the mating *Streptococcus* E25-3 x *E. faecalis* JH2/2, (6) *Streptococcus* E31-2, (7) transconjugant from the mating *Streptococcus* E31-2 x *E. faecalis* JH2/2, (8) *Streptococcus* E37-4, (9) transconjugant from the mating *Streptococcus* E37-4 x *E. faecalis* JH2/2, (10) *Streptococcus* E38-2, (11) transconjugant from the mating E38-2 x *E. faecalis* JH2/2, (12) *Streptococcus* E23-4, (13) *Streptococcus* E24-2, (14) *Streptococcus* E33-3.

6.5 Discussion

In the course of this study, 12 isolates were identified that were resistant to erythromycin and to tetracycline (Table 6.1). Four of these could transfer both resistance genes to an *Enterococcus faecalis* JH2/2 recipient strain (Table 6.1). In each case, the co-transferred genes were *erm*(B) and *tet*(M). The conjugative transposon Tn*1545* has previously been shown to be responsible for the transfer of tetracycline and erythromycin resistance, this element also contains the kanamycin resistance gene *aphA*-3 (276). The ends of Tn*1545,* which contain the *int* and *xis*

genes, are almost identical to those of Tn*916* (237) and, like Tn*916*, Tn*1545* also contains the *tet*(M) gene. Consequently, in order to test for the presence of a Tn*1545*-like element in the strains isolated, the parents and transconjugants were subjected to a Southern blot analysis against *aphA*-3, *tet*(M), the *int* and *xis* genes of Tn*916* and pAM120. The blots showed that in all the strains that contained a transferable element, the *aphA-3*, *tet*(M), *erm*(B) and *int/xis* genes were present and therefore are likely to be linked. No PCR products or hybridisation to the recipient DNA was observed (Fig 6.1 lane 1), indicating that a Tn*1545*-like element was responsible for the transfer.

Some of the donors did not transfer any of their resistance genes, although hybridisation analysis indicated that they were likely to be contained on a Tn*1545*-like element. It is known that the integration site of conjugative transposons has an effect on the transfer frequency of the elements (237). Therefore, the non-transferors may be inserted into a site from which the excision frequency is low. Other possibilities for non-transfer include: 1) that the conjugative transposon has a mutation in a gene essential for mobility or 2) essential host factors may be absent in some strains. This last event has been observed in *Lactococcus lactis*, which can act as a recipient of Tn*916*, however subsequent transfer of the conjugative transposon to another host does not take place implying that *L. lactis* is deficient for a factor required for conjugative transposition (29).

The Tn*916*-Tn*1545* family of elements has been well established in a variety of Gram-positive and Gram-negative organisms (51, 237, 267). The Tn*1545*-like element is identified in this study from viridans group streptococci (two *S. parasanguis*, one *S. salivarius* and one *S. mitis*, according to the partial 16S rRNA sequencing) for the first time. This transposon is very common in pathogenic streptococci such as *S. pyogenes* (101) and *S. pneumoniae* (174, 185, 267), but the association between erythromycin and tetracycline resistance does not seem to appear in other groups of streptococci (61) and there are few reports of this association in viridans group streptococci (293). In this last study, they showed the presence of *tet*(M), *erm*(B) and *int/xis* genes by PCR in *S. bovis*. However, further investigation is required to confirm the presence of Tn*1545* since a linkage of the *erm* and *tet* genes on the same element was not shown, and it is known that *int* and

xis genes can be found not linked to antibiotic resistance genes (144) or some strains resistant to erythromycin (*erm*(B)) and tetracycline (*tet*(M)) may carry the composite element Tn*3872* or one similar to it (174). This current study is the most complete investigation of the presence of Tn*1545* in VGS, as it has proved linkage between the tetracycline, erythromycin and kanamycin resistance genes in these organisms. The presence of a Tn*1545*-like element in oral streptococci is not surprising since Tn*916*-like elements have been shown to be transferred within a model oral biofilm between different oral streptococci (243), demonstrating that they are responsible for harbouring and disseminating mobile elements.

This study has revealed the presence of at least two different Tn*1545*-like conjugative transposons (Fig 6.1); transconjugants E25-3, E38-2 and E31-2 contained *erm*(B) and *tet*(M) on the same *Hind*III restriction fragment, whereas transconjugant E37-4 contained *erm*(B) and *tet*(M) on different *Hin*dIII restriction fragments. In the transposon detected in *S. pneumoniae* by Caillaud *et al.* in 1987 (33), the tetracycline and erythromycin resistance genes were separated by a *Hin*dIII site as well as the kanamycin and erythromycin resistance genes, furthermore the tetracycline resistance gene contains one *Hin*dIII restriction site.

When *int/xis* is used to probe *Hin*dIII digested DNA the number of bands reflects the number of copies of the transposon since it is responsible for the translocation of Tn*916* and is present in only one copy in Tn*916*-Tn*1545* family of transposons (188, 237). The hybridisation showed that all of the isolates tested possessed at least one copy of a Tn*1545*-like element (they all have one band only when probed with *int/xis*) and in lanes 7 and 11 the transconjugants received two copies of the transposon since they have two bands when probed with *int/xis* (Fig 1.9). The presence of multiple copies of the transposon after filter-mating has already been observed among conjugative transposons (207). Although the Tn*916*-Tn*1545* family of elements is not replicative, the presence of a second copy might be due to the intracellular transposition of Tn*916* from one of two daughter chromosomes after passage of the replication fork followed by insertion into a chromosomal region that has not yet replicated (237). Another explanation might be the co-transfer or the independent transfer of Tn*916* copies during a mating event (51).

The transfer frequencies obtained in this study were similar to the ones previously found for Tn*916*-like elements, that is from 10^{-9} and 10^{-4} transconjugants per donor (51).

No isolates with the *mef* gene only were found to be resistant to tetracycline, although a previous study found that most of the erythromycin-resistant oral streptococci of the MLS and M phenotype were also resistant to tetracycline (101). The mechanisms behind the rapid dissemination of the *mef* gene are being revealed. It could be due to the clonal expansion of one strain, since some studies have shown a link between the serotype and the presence of this gene (234), or to horizontal transfer. In one study, the *mef* gene was shown to be transferable between different species in the presence of DNAses, indicating that transfer is by conjugation or by transduction (164). Indeed, some *mef* genes have recently been found to be contained within a novel transposon Tn*1207.3* (259) or within the Tn*2009* element consisting of the *mef* gene inserted into a Tn*916*-like transposon (68).

In this study, it was demonstrated that there is variation in the genetic organisation of the Tn*1545*-like elements and that these elements are widespread in the oral cavity. Linkage of multiple antibiotic resistance genes on the same mobile element is of public health importance, because use of any of the antibiotics to which the element confers resistance selects for retention of the transposon and, accordingly, multiple antibiotic resistance genes. In the oral microbiota, interspecies exchange of resistance genes combined with antibiotic selection pressure, could select for more pathogenic multidrug-resistant bacteria.

Chapter 7

Identification of the Genetic Support for *tet*(W)

7.1 Abstract

There is considerable potential for genetic exchange between bacteria from different habitats. Recently, a new tetracycline resistance gene, *tet*(W), was isolated from *Butyrivibrio fibrisolvens*, a rumen bacterium. In the current study, *tet*(W) has been isolated from the oral cavity for the first time and was found to be the second most common tetracycline resistance gene after *tet*(M). One *Rothia* strain carrying *tet*(W) was further investigated. The gene was cloned into pUC18 and the recombinant plasmid sequenced. The plasmid contained an insert of approximately 13-kb, the sequence of which shows that *tet*(W) is linked to two different transposases, and located between two identical copies of the *mef* gene. Further PCR amplifications for this region of DNA on other oral isolates containing *tet*(W) demonstrated that this structure accounted for many of the genetic supports for *tet*(W) in the oral environment.

7.2 Introduction

The first *tet*(W) gene was identified from the bovine rumen anaerobe *Butyrivibrio fibrisolvens* and subsequently in human faecal anaerobes, pigs and in a facultative anaerobe *Arcanobacterium pyogenes* (17, 21, 264). In *B. fibrisolvens tet*(W) is contained within a large conjugative transposon, Tn*B1230* (17) which is capable of high frequency conjugative transfer among *B. fibrisolvens* species. In *A. pyogenes*, *tet*(W) is associated with a *mob* gene, not found in Tn*B1230,* and was capable of conjugative transfer at low frequency (21).

Commensal bacteria have often been shown to serve as reservoirs of antibiotic resistance genes eg those of the intestinal tract of humans and animals (17, 180, 264)

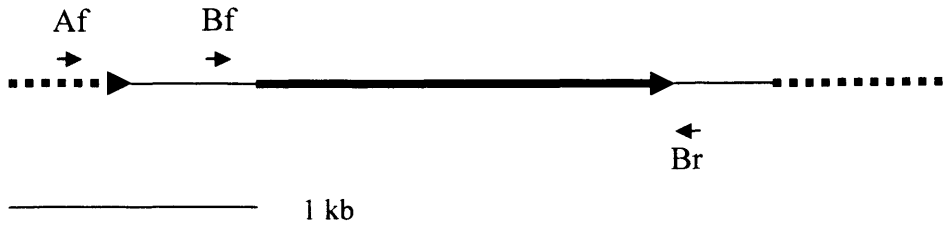
and viridans group streptococci of the upper respiratory tract of humans (31, 340). The presence of different types of elements carrying *tet*(W) among commensal rumen bacteria is clear evidence of their likely role in the expansion, dissemination, and preservation of antibiotic resistance genes and also in the evolution of those genes. By determining the genetic support of *tet*(W) in the oral cavity, we wanted to get a better understanding of the origin of this gene as well as the reason for its wide dissemination in this habitat.

7.3 Materials and Methods

The methods for sampling and detection of *tet*(W) have been described previously (see chapters 3 and 4).

PCR. In order to determine if the genetic support of the *tet*(W) genes we observed in our oral samples were similar to the *tet*(W) gene from *Butyrivibrio fibrisolvens* we carried out PCR amplifications using specific primers for the whole gene isolated from Tn*B1230* (primers Bf and Br Fig 7.1) as well as primers including the whole gene and the upstream region specific for Tn*B1230* (primers Af and Br Fig 7.1). Both sets of primers were provided by Brunel *et al.* (Appendix 3) and the PCR conditions were as follows, one cycle of 5 min at 94°C, 60 s at 50°C, 2 min at 72°C was performed followed by 29 cycles of 60 s at 94°C, 60 s at 50°C, and 2 min at 72°C with a final extension at 72°C for 10 min.

Figure 7.1: Position of the *tet*(W) primers compared with the whole gene isolated in Tn*B1230* from *Butyrivibrio fibrisolvens*



This diagram represents part of Tn*B1230*-containing *tet*(W) (179). The *tet*(W) gene is represented by the thick black line, two putative nitroreductases upstream and downstream of *tet*(W) are represented by dashes. Primers Af, Bf and Br (Appendix 3) were used in two different PCR reactions to compare *tet*(W)-positive isolates found in this study with *tet*(W) from Tn*B1230.* The arrows show the likely direction of transcription.

Cloning. One *Rothia* isolate containing *tet*(W) was selected for cloning (isolate T40.1 in Table 7.1). The genomic DNA was extracted (see chapter 2.3) and the concentration of DNA obtained was approximately 25 ng/µl, it was then partially digested with *Mbo*I (Biolab). For the digestion reaction, 34 µl of DNA was mixed with 4 µl of Buffer (10 x Buffer 3, Biolab), 2 µl of enzyme (5U/µl), in a total volume of 40 µl; the digestion was repeated twice at 37°C at two different times, one for 10 min and another for 15 min. Both digestions were pooled and run into a gel and the region between 4- and 10-kb was cut from the gel and purified using a gel extraction kit (see chapter 2.10.3). Ligation into pUC18 and transformation into DH5α (Invitrogen) were performed as stated in chapters 2.10.4 and 2.10.5 respectively. Transformants containing *tet*(W) were selected for on plates containing ampicillin (50 µg/ml) and tetracycline (5 µg/ml).

Subcloning. Part of the insert (around 6 kb downstream *tet*(W)) could not be sequenced and so was digested and subcloned. The plasmid containing the insert which conferred resistance was extracted as stated in chapter 2.10.1 and the final concentration obtained was around 50 ng/µl. For the digestion reaction 5 µl of the

plasmid-containing the insert was digested with two enzymes: 2 µl of *Hin*dIII (20 U/µl, Biolab) and 2 µl of *Eco*RI (20 U/µl, Biolab), and the reaction was mixed with 2 µl of Buffer (10 x Buffer 2, Biolab), 9 µl of dH_2O in a total volume of 20 µl and incubated at 37°C for 60 min. The digestion was run into a gel and according to the data already obtained it was deduced that bands of 2 and 5 kb were the bands of interest (Fig 7.2) and thus were cut from the gel and purified using a gel extraction kit (see chapter 2.10.3). The vector, pUC18, was also digested with both enzymes, the mixture was as follows: 10 µl of vector was mixed with 3 µl of *Hin*dIII (20 U/µl, Biolab), 3 µl of *Eco*RI (20 U/µl, Biolab), 10 µl of buffer (10 x Buffer 2, Biolab), 74 µl of dH_2O in a total volume of 100 µl, the digestion was incubated for 2 hrs at 37°C. Subsequent plasmid dephosphorylation, ligation and transformation reactions were performed as stated in chapters 2.10.2, 2.10.4 and 2.10.5 respectively.

Sequencing. Part of the insert of pPPMW (approximately 7 kb) was sequenced by the Lark Technology Inc (Takeley, UK) and the remainder of the insert was subcloned into pUC18 and sequenced using the universal primers M13F and M13R (Appendix 3) as stated in chapter 2.7. Subsequent primers were designed using Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The sequencing was performed on both strands and was compared to those in public sequence repositories (GenBank) using the basic local alignment search tool (BLAST) (6).

Comparison of *tet*(W) organisation. The different *tet*(W) isolates were compared by PCR using different sets of primers (Fig 7.3 and Appendix 3), the primers were designed as stated above. The PCR conditions were as folows 35 cycles of 60 s at 94°C, 60 s at 64°C and 180 s at 72°C with a final extension at 72°C for 5 min. Some of the PCR results were confirmed, where required, by DNA sequencing (see chapter 2.7).

Mating experiments. The *Rothia* sp. from which *tet*(W) was cloned (isolate T40.1) was tested for its ability to transfer the *tet*(W) gene to *Enterococcus faecalis* JH2-2 which is resistant to rifampicin (see chapter 2.8). The transconjugants were selected on tetracycline (5 µg/ml) and rifampicin (25 µg/ml) containing agar.

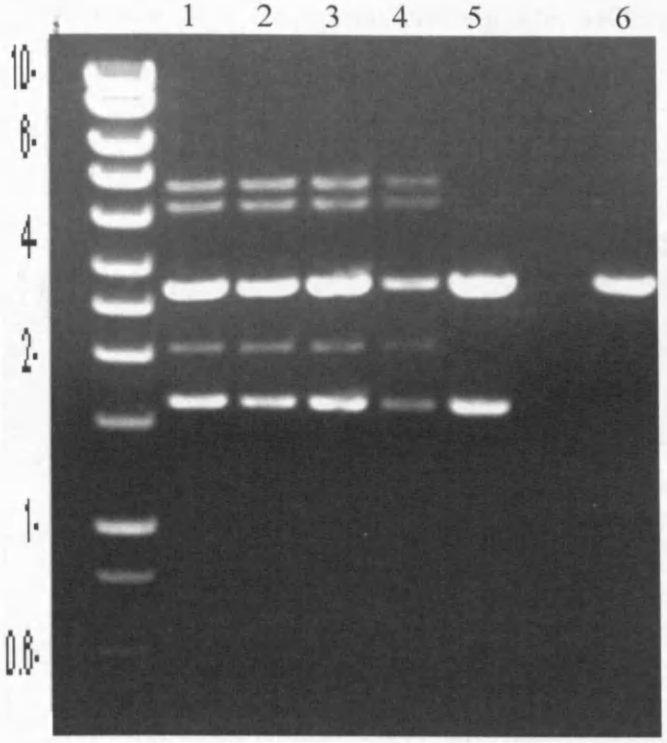
Stability assay. To study the stability of the insert in *E. coli*, the clones were grown overnight in LB broth (Appendix 1) containing ampicillin (50 μg/ml), serial diluted and plated onto ampicillin-containing plates and incubated for 18 hrs at 37°C. Plates containing around 50 single colonies were replicated onto a set of ampicillin plates only (50 μg/ml) and a set of ampicillin and tetracycline plates (5 μg/ml). The same experiment was performed with the original strain T40.1, the only difference was that it was grown overnight in BHI broth (Oxoid) without antibiotics.

7.4 Results

7.4.1 DNA sequence analysis of *tet*(W) cloned from *Rothia* sp.

The flanking regions of the *tet*(W) genes found in this study were compared, by PCR, with the flanking regions of *tet*(W) isolated from Tn*B1230* from *Butyrivibrio fibrisolvens*. PCR with primers Bf-Br and Af-Br (Fig 7.1) yielded products in 11 isolates out of 31 containing *tet*(W), however when these strains were re-tested it was found that the *tet*(W) gene was lost. The stability of the *tet*(W) gene in these strains could not be measured easily as the organisms all contained another *tet* gene. This could be due to recombination between the repeated flanking regions. Unfortunately no more work could be performed on these strains. Out of the 20 remaining isolates containing *tet*(W), one *Rothia* sp. (isolate T40-1 in Table 7.1) was further investigated. The *tet*(W) gene was cloned into pUC18 (see materials and methods). Four clones were isolated on tetracycline-containing plates (5 μg/ml) and tested by PCR for the presence of *tet*(W) and all produced a product of the expected size (data not shown). The restriction pattern obtained with *Hin*dIII and *Eco*RI was identical in each, showing that an insert of 13 kb had been cloned (Fig 7.2 lanes 1 to 4). One plasmid was chosen for further study and designated pPPMW.

Figure 7.2: Restriction pattern of pPPMW and pPPMW-1



Lanes 1 to 4 contain DNA from the 4 clones containing *tet*(W), pPPMW, lane 5 contains plasmid DNA that has spontaneously lost the *tet*(W) gene, pPPMW-1, and lane 6 contains pUC18. The samples were double digested with *Hin*dIII and *Eco*RI. The 1-kb DNA ladder (Biolabs) was used as the molecular size marker and the band size are written in kilobase on the left hand side of the gel.

The DNA sequence of pPPMW was determined (Table 7.1, Fig 7.3). Ten *orfs* were identified of which only *orf4*, which has sequence identity with the end of the asparate/ornithine carbamoyltransferase, has the same direction of transcription as *tet*(W) (Fig 7.3). *Orf5* is homologous to the transposase of the IS*30* family and it is associated with a 38 bp inverted repeat (Table 7.1). *Orfs6* and *7* are almost identical to the IS*1081* transposase of the IS*256* family; however the gene coding for IS*1081* in pPPMW has been split by a mutation which introduces a stop codon (Fig 7.3). Two additional ORFs potentially involved in determining resistance to macrolides were found upstream and downstream of *tet*(W) and were homologous to the *mef*(A) gene (Table 7.1). Downstream of *orf1* there are two ORFs, *orf2* and *orf3* that are homologous to the ATPase components of the ABC transporter family. However the gene in pPPMW has been split by the insertion of 34 nucleotides leading to a

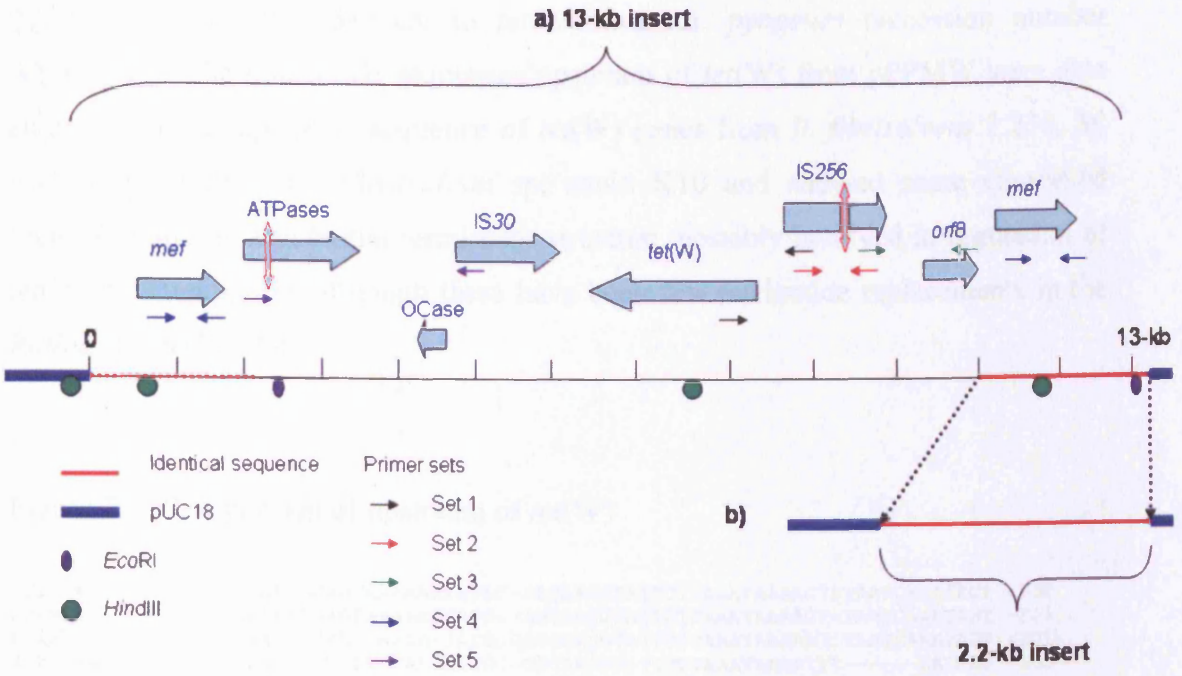
mutation and the insertion of a stop codon (Table 7.1 and Fig 7.3). Moreover, the presence of a ribosomal binding site within these 34 nucleotides suggests that the second orf, *orf3,* is functional.

Table 7.1: Closest relatives to the *orfs* flanking *tet*(W) of *Rothia* sp.

ORF	Length of predicted protein	G+C Content	Closest relative in the database	Source organism	Likely Function of protein	% Identitiy	E value
orf1	413 aa	58%	*mef*	*Strep*	macrolide-efflux protein	42%	1e-75
orf2	74 aa	51%	ATPase	*Strep*	ATPase components of ABC transporters	68%	2e-14
orf3	403 aa	56%	ATPase	*Strep*	ATPase components of ABC transporters	60%	e-137
orf4	97 aa	62%	end of asparate/ ornithine carbamoyltransferase	*Enteroc/ Lactoc*	asparate/ornithine binding domain	62%	2e-15
orf5	403 aa	74%	IS*30* transposase	*Strep*	putative family of transposase	30%	1e-34
tet(W)	524 aa	53%	*tet*(W)	*Butyri*	tetracycline resistance protein	97%	0.0
orf6	224 aa	62%	IS*256* transposase	*Mycob*	putative family of transposase	83%	2e-91
orf7	148 aa	61%	IS*256* transposase	*Mycob*	putative family of transposase	78%	2e-57
orf8	204 aa	58%	putative protein	*Burkhol*	putative protein	30%	> 5
orf9	428 aa	58%	*mef*	*Strep*	macrolide-efflux protein	42%	1e-86

aa = amino acids, *Strep* = *Sreptococcus* spp., *Enteroc/Lactoc* = *Enterococcus/Lactococcus* spp., *Butyri* = *Butiryvibrio fibriosolvens*, *Mycob* = *Mycobacterium avium*, *Burhold* = *Burkholderia ambifaria.*

Figure 7.3: Genetic support of *tet*(W) in an oral *Rothia* sp.



Schematic diagram of the deduced genetic organisation of a) the whole 13-kb insert contained in pPPMW and b) the deletion derivative (pPPMW-1) after the spontaneous loss of *tet*(W). The likely direction of transcription of the genes is shown by the arrowed boxes. The vertical arrows represent the disruption of the ORF: the ORF coding for ATPases is disrupted by the insertion of 34 extra nucleotides and there is a point mutation in IS*256* leading to the introduction of a stop codon. Highlighted in red are the identical sequences found in the deletion derivative and the right end of the 13-kb insert (the sequences are 100% identical at the nucleotide level), and this sequence is also repeated and 99% identical at the nucleotide level in the left end of the 13-kb insert (highlighted in red dashes). A series of primers were designed to amplify the region between *tet*(W) and IS*256* (set 1), IS*256* (set 2), the region between IS*256* and downstream *orf8* (set 3), *mef* (set 4), and the region between ATPases and IS*30* (set 5).

The nucleotide sequence of *tet*(W) from pPPMW was compared with other *tet*(W) genes, and it was 97% identical to *tet*(W) from Tn*B1230* (accession number AJ222769) and 91% identical to *tet*(W) from *A. pyogenes* (accession number AY049983). The nucleotide sequences upstream of *tet*(W) from pPPMW were also aligned with the upstream sequence of *tet*(W) genes from *B. fibrisolvens* 1.230, *M. multiacidus* P208, and *Clostridium* sp. strain K10 and showed some conserved regions including a potential terminator structure, possibly involved in regulation of *tet*(W) by attenuation, although there have been few nucleotide replacements in the *Rothia* strain (Fig 7.4).

Figure 7.4: Comparison of upstream of *tet*(W)

```
1.230W          AGAAAAGGAGTAAAAAATATGC-GGCAAGGTATTCTTAAATAAAACTATAATCAAATAGT -204
208W            AGAAAAGGAGTAAAAAATATGC-GGCAAGGTATTCTTAAATAAAACTATAATCAAATAGT -204
K10W            AGAAAAGGAGTAAAAAATATGCCGGCAAGGTATTCTTAAATAAAACTATAATCAAATAGT -203
RothiaW         -TGAGGAGTCTACCAAATATGC-GGCAAGGTATTCTTAAATAAAATTT------GATAAT -185
                   *   *  **  ******** ********************** *        *** *

1.230W          GGGAACAAAGGATTATGATAGCTCCTTTTGTAGGGGCTTAGTTTTTTGTACCCAATTTAA -164
208W            GGGAACAAAGGATTATGATAGCTCCTTTTGTAGGGGCTTAGTTTTTTGTACCCAATTTAA -164
K10W            GGGAACAAAGGATTATGATAGTTCCTTTTGTGGGGGCGGGATTTTTTGTACCCAATTTAA -163
RothiaW         GGGCGCAAAA----ATGATTGCCCCT--TGCAGGGGCTTAGTTTTT-GTACCCAATTTAA -152
                ***  ****     ***** *  ***  **  *****    ***** ************

1.230W          GAATACTTTTGCCTTATCAATTTTGACATATCCCCAAAAACAGCAATCACAAACAGGTGT -104
208W            GAATACTTTTGCCTTATCAATTTTGACATATCCCCAAAAACAGCAATCACAAACAGGTGT -104
K10W            GAATACTTTTGCCTTATCAATTTTGACATATCCA-AAAAACAGCAGTCACAAATAGGTGT -104
RothiaW         GAATACTTTTGCCTTTTTAGT----AAATATCGTGACGGACAGCGGCT-CATGTAAGC-- -99
                *************** * * *    * *****   *   *****     **   * *

1.230W          ATGCTGTATATGTGTATGTCCGCAACTTATAATCCCCAGTGGTAAAAGTATTTTACTGCT -44
208W            ATGCTGTATATGTGTATGTCCGCAACTTATAATCCCCAGTGGTAAAAGTATTTTACTGCT -44
K10W            ATGCTGTATATGTGTATGTCCGCAAATTATAATCCCCAGTGGTAAAAGTATTTTACTGCT -44
RothiaW         -CGTCATACTTCTGTTTGTCCG-AAGTCATGATCCCCAGCGGTAAAAGTATTA-GCCGCT -42
                  *   **  * *** ****** ** * ** ******** ************   * ***

1.230W          GGGGATTTTTATGCCCTTTGGGGCTGTAAAGGGAGGACAATCACATGAAAATAATCAATA 16
208W            GGGGATTTTTATGCCCTTTGGGGCTGTAAAGGGAGGACAATCACATGAAAATAATCAATA 16
K10W            GGGGATTTTTTGTGCCCTTTCGGGCTGTAAAAGGAGGACAATCACATGAAAATAATCAATA 16
RothiaW         GGGGATTTTTTGCGCCCATTTGGGCCTTGTATGGAGGATAG--ACATGAACATTATCAATA 16
                **********  **** ** ****  *  * ****** *   ******* ** *******
```

The CLUSTLAW program was used to align the nucleotide sequences upstream of the *tet*(W) gene from T40-1 with the upstream sequence of *tet*(W) genes from *B. fibrisolvens* 1.230 (GenBank accession number AJ222769), *M. multiacidus* P208 (GenBank accession number AY603069), and *Clostridium* sp. strain K10 (GenBank accession number AY601650). The solid arrows indicate the inverted repeats, a poly(T) region which could act as a rho independent transcriptional terminator, ribosome binding sites (GGAGGA), and the ATG start codon are highlighted in green, blue, and red respectively.

7.4.2 The genetic supports for *tet*(W) genes in oral isolates

From the sequence obtained, primers were designed upstream and downstream of *tet*(W) in pPPMW to compare the organisation of the flanking region of *tet*(W) with the other *tet*(W)-containing isolates. The binding sites of the primers are shown in Figure 7.3. A total of 20 isolates were tested, including the original strain T40-1 from which the *tet*(W) gene was cloned. According to the PCR results with the different sets of primers, the isolates were divided into 3 groups (Table 7.2). Group 1 included four isolates that, according to the PCR results, had a similar genetic structure surrounding the *tet*(W) gene to the Rothia T40.1 strain. Group 2 included eight isolates that, according to the PCR results, had homology only with part of the sequence contained within pPPMW. Finally Group 3 contained eight isolates that did not have any homology with pPPMW and so did not give any PCR products with the primer sets used although two out of these nine isolates had the *mef* gene and so gave a positive PCR product with the primer set 4 (Table 7.2 Group 3).

Table 7.2: Study of the genetic support of *tet*(W) isolated from oral bacteria by PCR

Groups	Tet W isolate	Other *tet* genes	PCR with primers Bf/Br and Af/Br	PCR with primers designed from the sequence of the new element					Genus
				Set1	Set2	Set3	Set4	Set5	
Group 1	T29.1	none	-	+	+	+	+	+	Rothia
	T40.1	none	-	+	+	+	+	+	*Rothia*
	T54.2	none	-	+	+?	+	+	+	*Rothia*
	T59.3	none	-	+	+	+	+	+	*Strep*
Group 2	T22.3	*tet*(M)	-	+	+	-	+	+?	*Rothia*
	T56.4	none	-	+	+	-	+	+?	*Rothia*
	T60.2	*tet*(M)	-	+	+	-	+	+	*Staph*
	T60.9	none	-	+	+	-	+	+	*Actino*
	T44.5	none	-	+	+	-	-	+	*Lactob*
	T48.7	none	-	+	+	-	-	+	*Lactob*
	T43.7	none	-	+	+	-	-	-	*Actino*
	T51.1	none	-	+	+	-	-	-	*Lactob*
Group 3	T27.5	*tet*(M)	-	-	-	-	-	-	*Staph*
	T32.6	*tet*(M)	-	-	-	-	-	-	*Actino*
	T34.4	*tet*(L)	-	-	-	-	-	-	*Actino*
	T39.3	none	-	-	-	-	-	-	*Actino*
	T46.6	none	-	-	-	-	-	-	*Actino*
	T50.1	none	-	-	-	-	+	-	*Rothia*
	T51.6	none	-	-	-	-	+	-	*Rothia*
	T55.5	none	-	-	-	-	-	-	*Actino*

All the *tet*(W)-containing isolates found in this study were compared by PCR to identify the genetic support of this antibiotic resistance gene in oral bacteria. We identified 3 different groups. Group 1 contained isolates related to the whole of the putative new element identified in this study from T40-1, highlighted in red. Group 2 contained isolates related to part of the putative new element. Finally Group 3 contained isolates with no similarities with TnB*1230* or the putative new element.

+? = a PCR product was obtained with these isolates and the sequencing of the ends indicated that the expected PCR product had been amplified however the PCR product had a higher molecular weight than the positive control (see explanation chapter 7.4.2).

Strep = *Streptococcus*, *Staph* = *Staphylococcus*, *Actino* = *Actinomyces*, *Lactob* = *Lactobacillus*.

Furthermore, one isolate from Group 1 set 2 (Table 7.2 isolate T54-2) and two isolates from Group 2 set 5 (Table 7.2 isolates T22-3 and T56-4) yielded a bigger PCR product than the corresponding positive PCR control (pPPMW); these samples were subsequently sequenced. The PCR product from isolate T54-2 using primer set 2 was 1,285 bp long instead of 1,078 bp in pPPMW and was fully sequenced. It exhibited high sequence identity with the transposase belonging to the IS*256* family sequenced in pPPMW (97% nucleotide identity); however the sequences diverge at 1,144 bp. The reason for the amplification of a larger product from T54-2 is the presence of an inverted repeat (highlighted in red in Fig 7.5) which can act as a primer binding site.

Figure 7.5: Comparison of PCR products obtained from pPPMW and T54-2 isolate using primer set 2

```
                ****************************** *********   ********  *******

IS256/pPPMW     CCAACTCAGCGCATAACCCACCCCGAAGGACACAAAACGATTACACCACTCCACAGGACT 1138
IS256/T54-2     CCAACTCAGCGCATAACCCAACCCGAAGGACACAAAACGATTACACCACTCCACAGGACT 1140
                ******************* ************************************

IS256/pPPMW     TGACCCGCCAA---GCAGCCACCTCGCGAAATTCAGCACGAGCACGCCCTCCCGGGTATG 1195
IS256/T54-2     TGACCACCGGATGCGTAGGGTTTTCCGGAGTCTAGGGACTTTAGGGAGATGCCGCGCTTG 1200
                *****  *  *   * **     **  **   *  * **      *   * *** *  **

IS256/pPPMW     GGGCTCATGCCTCGTGCGAGTGATGAGAATCTTCGGGAATGCATCCCCGATGGATAGAAT 1255
IS256/T54-2     ACCAGCTTGGACTG-GAAAACGTCGAGCA-CTGCCAGGGCGCGCTCTTCGGCGTTTGCCT 1258
                     * **    * *  *  *  *** * ** *  *   **   *      * * *  *

IS256/pPPMW     CGGCGCAATCTCCCTCTCGAGCGTCGA 1282
IS256/T54-2     TGATGAGGATCTTGTTGACCCCACTGA 1285
                 *  *         *     *   **
```

The arrows represent the forward (5'-CAGTGGGGTCAACAAGATCC-3') and the reverse primers (5'-AGTGGGGGTTCCTCCTGTT-3') (Fig 7.3 set 2).
Highlighted in red are the inverted repeats found in the bigger PCR product amplified from T54-2. Highlighted in blue is the nucleotide sequence found in the bigger PCR product and that differ from the sequence in pPPMW.
The particularity of the larger product is that it is flanked by two inverse repeat sequences of 21 nucleotides allowing the forward primer to act as a reverse primer hence the larger PCR product obtained from isolate T56-4.

The bigger PCR products from isolates T22-3 and T56-4 using primer set 5 were partially sequenced (2,034 bp were sequenced out of a total PCR product of around 3,500 bp) and they exhibited high sequence identity with the ATPase and the ornithine carbamoyltransferase sequenced in pPPMW (99% and 100% nucleotide identities respectively), however the nucleotide region between *orf3* and *orf4* could not be sequenced and may be due to the presence of repeated sequences in this region.

7.4.3 Stability of *tet*(W) in *E. coli* and *Rothia* sp.

When grown in the absence of tetracycline, all of 50 colonies tested had lost their resistance to the drug. Plasmids were prepared from 5 sensitive colonies and digested with *Hin*dIII and *Eco*RI, revealing that an 11-kb sequence had been deleted (lane 5 Fig 7.2) in each of the 5 colonies tested, this new plasmid was named pPPMW-1. It can be seen from Figure 7.2 that there is a mixture of intense and faint bands in the

lanes containing plasmid from tetracycline-resistant clones. The intense bands correspond to those remaining after loss of tetracycline resistance indicating that there is a mixture of full length plasmids (pPPMW) and deletants (pPPMW-1). When *Rothia* strain (T40.1) was grown in the absence of tetracycline no loss of *tet*(W) was observed after 21 passages, showing that the gene is relatively stable in its original host.

The complete nucleotide sequence of the insert of pPPMW and pPPMW-1 was obtained. pPPMW-1 was found to be a deleted version of pPPMW; it consists of *orf9* and part of the unknown *orf8* (Fig 7.3).

No transfers of *tet*(W), from *Rothia* sp. (T40.1) to JH2/2, were detected under the conditions used (see materials and methods), the experiment was performed twice.

7.5 Discussion

7.5.1 The genetic support of *tet*(W) in pPPMW

The *tet*(W) gene was shown to be the second most common tetracycline resistance gene identified from the oral cavity (see chapter 4). The genetic support of the gene was compared with the genetic support of the original *tet*(W) gene from *Butyrivibrio fibrisolvens* by PCR using specific primers for the upstream region of the gene contained in Tn*B1230*; only 11 out of 31 isolates carrying *tet*(W) yielded positive PCR products suggesting a similar organisation as in TnB*1230*. No further work could be carried out on these organisms because of the loss of *tet*(W) following retrieval of the strains. The genetic support of *tet*(W) was characterised in detail in one of the 20 remaining isolates containing *tet*(W) (*Rothia* T40-1 Table 7.1). The *tet*(W) from this strain was cloned into pUC18 to generate pPPMW DNA sequence analysis of this insert showed that *tet*(W) was flanked by two copies of the *mef* gene including part of an unknown open reading frame *orf9*, two putative transposases belonging to two different families of insertion sequences (IS*30* and IS*256*) and one ATPase downstream the *tet*(W) gene (Fig 7.3 a)).

7.5.1.1. Features of the transposases

One putative transposase from the IS*30* family was found downstream of *tet*(W), another incomplete transposase of the IS*256* family was located upstream of the tetracycline resistance gene. The presence of an inverted repeat upstream of the IS*30* transposase is likely to regulate the transcription of the element at the RNA level; the repeat sequence can form a loop preventing transcription of the transposase (56).

Members of the IS*30* family usually contain a single *orf* flanked by inverted repeats (IRs) in the range of 20-30 bp that exhibit significant homologies. Various IS*30* family members have been identified as part of composite transposons associated with antibiotic resistance genes eg in VRE strains (114), and in macrolide-resistant *Bacteroides fragilis* (228). Members of the IS*30* family of transposases have also been shown to be present in oral streptococci and *S. pyogenes* in several copies although it is not known whether they are associated with antibiotic resistance genes in these strains (169).

The IS*256*-family of transposases was originally found in *Mycobacterium* sp. (169), however it is not known whether this IS is functional in *Rothia* sp. since its organisation is slightly different due to a mutation which introduces a stop codon at position 675. In some IS sequences (eg IS*3*), translational frameshifting is used as a control mechanism for transposases expression (169). This could be the case in the IS*256*-like element from *Rothia* sp. and further work would be needed to confirm this event in our strain.

IS elements are frequent components of multi-resistant elements. The *tet*(W) gene isolated in this study is flanked by two transposases belonging to two different families of IS, IS*30* and IS*256*, therefore it might be part of a composite transposon allowing its dissemination in oral bacteria.

7.5.1.2 Features of the *mef* gene

The *mef* gene has recently been linked with two different tetracycline resistance genes. It was found to be co-transferred with *tet*(O) between strains of *S. pyogenes* as well as between *S. pyogenes* and *E. faecalis* JH2/2, however the mobile element was not identified (101). In a different study, a *mef* gene was shown to be inserted into a Tn*916*-like element containing *tet*(M), however this new composite element, Tn*2009*, could not be transferred (68). In the present study *tet*(W) was flanked with two copies of the *mef* gene, although no co-transfer was shown.

The *mef* gene contained in Tn*1270.1* and Tn*2009* is associated with a member of the ABC transporter family (68, 259), as is the case in the current study. An alignment of the deduced amino acid sequence of *mef* and the ABC transporters from Tn*2009*, Tn*1270.1* and pPPMW showed that the percentage identity was 40% and 58% respectively. The ABC transporter protein provides the energy required for the efflux of different substrates including macrolides and Streptogramin A (302) and thus might act as a dual efflux system with the *mef* gene.

7.5.1.3 Features of *tet*(W) and upstream of the gene in different isolates

The upstream region of *tet*(W) in pPPMW was compared with the upstream region of *tet*(W) from different isolates (Fig 7.4), and showed some conserved regions as already mentioned in Melville *et al.* (179). Overall, there is 78% sequence conservation upstream of the *tet*(W) genes from the different strains. The region upstream of the *Rothia* gene is more divergent that upstream of the other *tet*(W) genes (Fig 7.4). The presence of conserved regions highlights the likely importance of this sequence for the tetracycline resistance genes. Indeed, the sequence immediately upstream from the *tet*(W) gene was shown to be required for full expression of resistance (179) and the 17-bp inverted repeats, also highly conserved upstream of *tet*(O), *tet*(M), *tet*(S), *tet*(T) and *tet*(32) were shown to be important for regulating gene expression in *tet*(O) (315) and *tet*(M) (282). Moreover, this sequence conservation of *tet*(W) in the *Rothia* strain as well as upstream of the gene with the

previously studied *tet*(W) from bacteria from the rumen, porcine and human faeces is sign of a recent transfer of the gene between these different environments.

7.5.2 Stability of *tet*(W) in *E. coli* and *Rothia* sp.

When tetracycline sensitive deletant (pPPMW-1) was compared with the original clone (pPPMW) the regions remaining after deletion were the downstream and upstream sequence of *tet*(W), including one copy of the *mef* gene and part of an unknown ORF (*orf8*) (Fig 7.3). However, homologous recombination between these two almost identical copies of *mef* in pPPMW resulting in the loss of the *tet*(W) gene in pPPMW-1 is unlikely since the *E. coli* host strain, DH5α, used for cloning is a *recA* mutant. The basis of instability of this element in *E. coli* is not clear; it could be due to the action of the transposases that flank the *tet*(W) gene. The construction of mutants in these transposases would be useful to test this hypothesis. When *Rothia* T40.1 was grown in the absence of tetracycline, the *tet*(W) gene appeared very stable. This loss of stability of an element once it is cloned has already been observed with Tn*916* (96) and the Tn*4451*/Tn*4453* family of mobilisable transposons (2). In the case of Tn*4451*, the instability is due to the action of the transposase *tnpX*, if this is deleted, the transposon becomes stable whereas the instability in Tn*916* is thought to be due to the products of the *int* and *xis* genes.

7.5.3 The genetic support of *tet*(W) in oral isolates

In a total of 20 *tet*(W)-containing oral bacteria isolated and studied, different genetic supports for the *tet*(W) gene were identified: four *tet*(W)-containing oral bacteria were found to have related flanking DNA to the one sequenced in pPPMW including both transposases, the unknown *orf,* the *mef* gene and the ATPases, eight *tet*(W)-containing oral bacteria showed similarities with only part of the flanking DNA sequenced in pPPMW (they all contained IS*256* and some had the *mef* gene or the ATPases or both), and eight *tet*(W)-containing oral bacteria did not have any similarities with any of the flanking DNA and thus require further work. Further study is also needed to determine if the *Rothia tet*(W) is mobile and to continue the

sequencing upstream and downstream of the cloned region to determine whether this new element is part of a larger composite transposon.

This work is of importance since it shows that one antibiotic resistance gene, *tet*(W), can be contained within different mobile elements in the oral microbiota. The fact that the new putative element was identified in different genera in the oral microbiota is further confirmation that oral bacteria can transfer antibiotic resistance genes between isolates from different species and genera.

Chapter 8

Final Discussion

Because the oral bacteria form a community made up of different species and because they have the opportunity for contact with bacteria from other environments through the ingestion of food, vomiting and kissing, they are likely to play an important role as a reservoir of antibiotic-resistant bacteria and antibiotic resistance genes. This study showed that antibiotic-resistant bacteria and antibiotic resistance genes are present in the oral cavity and that oral bacteria may play an important role in the evolution and dissemination of antibiotic resistance genes.

The proportion of resistant bacteria in the oral cavity of healthy adults to commonly used antibiotics (tetracycline, erythromycin and amoxycillin) was shown to be important. Out of 60 samples screened, all of the individuals were found to harbour bacteria resistant to tetracycline and erythromycin and only 4 individuals did not have any cultivable bacteria resistant to amoxycillin. However no bacteria with acquired resistance to gentamicin or vancomycin, antibiotics of the last resort for some multidrug-resistant bacterial infections, were isolated during the study. This study illustrates how the use of antibiotics can exert a selection pressure in an environment, such as the oral microbiota, and lead to the establishment of a resistant population.

Out of two batches of 20 samples each, a representative 209 tetracycline-resistant bacteria were screened for the presence of tetracycline resistance genes. Most of the isolates carried tetracycline resistance genes encoding a ribosomal protection protein. The most common tetracycline resistance genes identified were *tet*(M), *tet*(W), *tet*(O) and *tet*(Q). The *tet*(M) gene had already been found to be the most common tetracycline resistance gene isolate from the oral microbiota (248) however *tet*(W) was isolated for the first time from this habitat. It was originally identified in a rumen bacterium, *Butyrivibrio fibrisolvens*, and was subsequently found in human faecal anaerobes, pigs and the animal pathogen, *Arcanobacterium pyogenes*. Moreover the

sequence conservation in these different *tet*(W) genes is sign of a recent transfer of the gene between these different environments.

Out of one batch of 20 samples, a representative 122 erythromycin-resistant isolates were screened for the presence of erythromycin resistance genes. A total of 28 isolates carried the *mef* gene while 14 isolates were resistant to erythromycin through the production of a methylase, *erm*(B). The remaining isolates with no identified erythromycin resistance genes were Gram-negative bacteria and are likely to be intrinsically resistant to the drug or to carry a non-specific *mtr* efflux pump. Most of the isolates with an identified erythromycin resistance gene belonged to the viridans group streptococci. These results agree with previous studies which have looked at the prevalence of the erythromycin resistance genes in pharyngeal samples and found that viridans streptococci are a reservoir of *erm*(B) and *mef* genes (9, 218, 340).

A Tn*1545*-like element was isolated for the first time from viridans streptococci. The Tn*916* and Tn1*545* family of conjugative transposons are responsible for the widespread dissemination of tetracycline and macrolide resistance in pathogenic bacteria, *S. pyogenes* and *S. pneumoniae* and therefore viridans streptococci might act as a reservoir for this type of element in the oral cavity. The Tn*1545*-like element isolated in this study exhibited a variation in its structure between different oral streptococci underlying the fact that these elements are not static.

One tetracycline resistance gene, *tet*(W), was isolated for the first time from cultivable oral bacteria and was shown to be the second most common tetracycline resistance gene after *tet*(M). By studying the genetic support of *tet*(W) we showed at least four different types of flanking regions in oral bacteria. Some *tet*(W) genes were flanked by two different IS elements and two copies of the *mef* gene. Some were flanked by only part of the new putative element including the IS*256* transposase, and some also had the *mef* gene while others had the *mef* gene plus the ATPase and ornithine carbamoyltransferase. Finally, a last group of *tet*(W) genes were not flanked by any of the sequences mentioned above.

These results are a reminder that antibiotics act upon the entire population exposed pathogenic as well as commensal. In the last few decades there has been a period of

strong selection pressure that has encouraged the evolution and dissemination of antibiotic-resistant organisms. The diversity of bacterial gene pools, the mobility of genes across species and genus boundaries, and the short generation times and large population sizes of bacteria make it even more difficult to curtail this spread of antibiotic resistance. As seen in this study an ongoing evolution is occurring as these bacteria cope with the ever-changing landscape in the environment, and although there has been an increased awareness about the consequences of overuse of antibiotics in humans as well as animals, some drastic changes are still needed to curtail the dissemination of resistance genes among bacteria. The study and discovery of new elements responsible for the spread of resistance is also of importance in gaining a better understanding about the origin of these elements.

References

1. Abraham EP, Chain E. 1940. An enzyme from bacteria able to destroy penicillin. Nature 3713:837.

2. Adams V, Lyras D, Farrow KA, Rood JI. 2002. The clostridial mobilisable transposons. Cell Mol Life Sci. 59(12):2033-43.

3. Addy M, Slayne MA, Wade WG. 1992. The formation and control of dental plaque--an overview. J Appl Bacteriol. 73(4):269-78.

4. Agerso Y, Guardabassi L. 2005. Identification of Tet 39, a novel class of tetracycline resistance determinant in *Acinetobacter* spp. of environmental and clinical origin. J. Antimicrob. Chemother. 55:566-569.

5. Alcaide F, Linares J, Pallares R, Carratala J, Benitez MA, Gudiol F, Martin R.. 1995. In vitro activities of 22 beta-lactam antibiotics against penicillin-resistant and penicillin-susceptible viridans group streptococci isolated from blood. Antimicrob Agents Chemother. 39(10):2243-7.

6. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

7. Aminov RI, Chee-Sanford JC, Garrigues N, Teferedegne B, Krapac IJ, White BA, Mackie RI. 2002. Development, validation, and application of PCR primers for detection of tetracycline efflux genes of Gram-negative bacteria. Appl Environ Microbiol. 68(4):1786-1793.

8. Andremont A, Gerbaud G, Courvalin P. 1986. Plasmid-mediated high-level resistance to erythromycin in *Escherichia coli*. Antimicrob Agents Chemother. 29:515-518.

9. Aracil B, Miñambres M, Oteo J, Torres C, Gómez-Garcés JL, Alós JI. 2001. High prevalence of erythromycin-resistant and clindamycin-susceptible (M phenotype) viridans group streptococci from pharyngeal samples: a reservoir of *mef* genes in commensal bacteria. J. Antimicrob. Chemother. 48: 592-594.

10. Arpin C, Canron MH, Maugein J, Quentin C. 1999. Incidence of *mef*(A) and *mef*(E) genes in viridans group streptococci. Antimicrob Agents Chemother. 43(9):2335-6.

11. Arthur M, Andremont A, Courvalin P. 1987. Distribution of erythromycin esterase and rRNA methylase genes in members of the family *Enterobacteriaceae* highly resistant to erythromycin. Antimicrob Agents Chemother. 31(3):404-9.

12. Ayoubi P, Kilic AO, Vijayakumar MN. 1991. Tn*5253*, the pneumococcal omega (cat tet) BM6001 element, is a composite structure of two conjugative transposons, Tn*5251* and Tn*5252*. J Bacteriol. 173(5):1617-22.

13. Bager F, Madsen M, Christensen J, Aarestrup FM. 1997. Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant *Enterococcus faecium* on Danish poultry and pig farms. Prev Vet Med. 31(1-2):95-112.

14. Bagg J, MacFarlane TW, Poxton IR, Miller CH, Smith AJ. 1999. Essentials of microbiology for dental students. Oxford University press Inc., NY.

15. Balsalobre L, Ferrandiz MJ, Linares J, Tubau F, de la Campa AG. 2003. Viridans group streptococci are donors in horizontal transfer of topoisomerase IV genes to *Streptococcus pneumoniae*. Antimicrob Agents Chemother. 47(7):2072-81.

16. Bantar C, Fernandez Canigia L, Relloso S, Lanza A, Bianchini H, Smayevsky J. 1996. Species belonging to the "*Streptococcus milleri*" group: antimicrobial susceptibility and comparative prevalence in significant clinical specimens. J Clin Microbiol. 34(8):2020-2.

17. Barbosa TM, Scott KP, Flint HJ. 1999. Evidence for recent intergenic transfer of a new tetracycline resistance gene, *tet*(W), isolated from *Butyrivibrio fibrisolvens*, and the occurrence of *tet*(O) in ruminal bacteria. Environ Microbiol. 1:53-64.

18. Bellido JLM, Guirao GY, Zufiaurre NG, Manzanares AA. 2002. Efflux-mediated antibiotic resistance in Gram-positive bacteria. Rev Medical Microbiol. 13(1):1-13.

19. Bentorcha F, Clermont D, de Cespedes G, Horaud T. 1992. Natural occurrence of structures in oral streptococci and enterococci with DNA homology to Tn*916*. Antimicrob Agents Chemother. 36(1):59-63.

20. Bertolla F, Simonet P. 1999. Horizontal gene transfers in the environment: natural transformation as a putative process for gene transfers between transgenic plants and microorganisms. Res Microbiol. 150(6):375-84.

21. Billington SJ, Songer JG, Jost BH. 2002. Widespread distribution of a Tet W determinant among tetracycline resistant isolates of the animal pathogen *Arcanobacterium pyogenes*. Antimicrob Agents Chemoter. 46(5):1281-1287.

22. Billot-Klein D, Gutmann L, Sable S, Guittet E, van Heijenoort J. 1994. Modification of peptidoglycan precursors is a common feature of the low- level vancomycin-resistant VANB-type Enterococcus D366 and of the naturally glycopeptide-resistant species *Lactobacillus casei*, *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, and *Enterococcus gallinarum*. J. Bacteriol. 176 (8):2398-2405.

23. Bingen E, Leclercq R, Fitoussi F, Brahimi N, Malbruny B, Deforche D, Cohen R. 2002. Emergence of group A *Streptococcus* strains with different mechanisms of macrolide resistance. Antimicrob Agents Chemother. 46:1199-1203.

24. Blondeau JM, Yaschuk Y. 1995. In vitro activities of ciprofloxacin, cefotaxime, ceftriaxone, chloramphenicol, and rifampin against fully susceptible and moderately penicillin-resistant *Neisseria meningitidis*. Antimicrob Agents Chemother. 39(11):2577-9.

25. Bonomo RA. 2000. Multiple antibiotic-resistant bacteria in long-term-care facilities: An emerging problem in the practice of infectious diseases. Clin Infect Dis. 31(6):1414-22.

26. Bonten MJ, Willems R, Weinstein RA. 2001. Vancomycin-resistant enterococci: why are they here, and where do they come from? Lancet Infect Dis.1(5):314-25.

27. Bonten MJ, Slaughter S, Ambergen AW, Hayden MK, van Voorhis J, Nathan C, Weinstein RA. 1998. The role of "colonization pressure" in the spread of vancomycin-resistant enterococci: an important infection control variable. Arch Intern Med. 158(10): 1127-32.

28. Bradford PA. 2001. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin Microbiol Rev. 14(4):933-951.

29. Bringel F, Van Alstine GL, Scott JR. 1991. A host factor absent from *Lactococcus lactis* subspecies *lactis* MG1363 is required for conjugative transposition. Mol Microbiol. 5(12):2983-93.

30. Brunel R, Scott KP. 2001. Distribution and degree of identity of the newly discovered tetracycline resistance gene *tet*(W). Unpublished data.

31. . Viridans group streptococci: a reservoir of resistant bacteria in oral cavities. Clin Microbiol Infect. 8(2):65-9.

32. Burrus V, Pavlovic G, Decaris B, Guedon G. 2002. Conjugative transposons: the tip of the iceberg. Mol Microbiol. 46(3):601-10.

33. Caillaud F, Carlier C, Courvalin P. 1987. Physical analysis of the conjugative shuttle transposon Tn*1545*. Plasmid. 17:58-60.

34. Canchaya C, Fournous G, Chibani-Chennoufi S, Dillmann ML, Brüssow H. 2003. Phage as agents of lateral gene transfer. Curr Opinion in Microbiol. 6:417-424.

35. Canchaya C, Proux C, Fournous G, Bruttin A, Brussow H. 2003. Prophage genomics. Microbiol Mol Biol Rev. 67(2):238-76.

36. Canu A, Malbruny B, Coquemont M, Davies TA, Appelbaum PC, Leclercq R. 2002. Diversity of ribosomal mutations conferring resistance to macrolides,

clindamycin, streptogramin, and telithromycin in *Streptococcus pneumoniae*. Antimicrob Agents Chemother. 46(1):125-31.

37. Casjens S. 2003. Prophages and bacterial genomics: what have we learned so far? Mol Microbiol. 49(2):277-300.

38. Catchpole MA. 1992. Sexually transmitted diseases in England and Wales: 1981-1990. Communicable Disease Review 2(1):1-7.

39. Celli J, Trieu-Cuot P. 1998. Circularization of Tn*916* is required for expression of the transposon-encoded transfer functions: characterization of long tetracycline-inducible transcripts reading through the attachment site. Mol Microbiol. 28(1):103-17.

40. Chadwick PR, Chadwick CD, Oppenheim BA. 1996. Report of a meeting on the epidemiology and control of glycopeptide-resistant enterococci. J Hosp Infect. 33(2):83-92.

41. Chain E, Florey HW, Gardner AD, Heatley NG, Jennings MA, Orr-Ewing J, Sanders AG. Penicillin as a chemotherapeutic agent. Lancet 2: 226-228. In Microbiology: A Centenary Perspective, edited by Wolfgang K. Joklik, ASM Press. 1999, p.112.

42. Charpentier E, Gerbaud G, Courvalin P. 1994. Presence of the Listeria tetracycline resistance gene *tet*(S) in *Enterococcus faecalis*. Antimicrob. Agents Chemoter. 38(10):2330-2335.

43. Charpentier E, Gerbaud G, Courvalin P. 1993. Characterization of a new class of tetracycline resistance gene *tet*(S) in *Listeria monocytogenes* BM4210. Gene 131(1):27-34.

44. Chiou CS, Jones AL. 1993. Nucleotide sequence analysis of a transposon (Tn*5393*) carrying streptomycin resistance genes in *Erwinia amylovora* and other gram-negative bacteria. J Bacteriol. 175(3):732-40.

45. Chopra I, Roberts M. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev. 65(2):232-60

46. Christie PJ. Type IV secretion: intercellular transfer of macromolecules by systems ancestrally related to conjugation machines. 2001. Mol Microbiol. 40(2):294-305.

47. Chuma T, Ikeda T, Maeda T, Niwa H, Okamoto K. 2001. Antimicrobial susceptibilities of *Campylobacter* strains isolated from broilers in the southern part of Japan from 1995 to 1999. J Vet Med Sci. 63(9):1027-9.

48. Chung WO, Werckenthin C, Schwarz S, Roberts MC. 1999. Host range of the *ermF* rRNA methylase gene in bacteria of human and animal origin. J Antimicrob Chemother. 43:5-14.

49. Clancy J, Petitpas J, Dib-Hajj F, Yuan W, Cronan M, Kamath AV, Bergeron J, Retsema JA. 1996. Molecular cloning and functional analysis of a novel macrolide-resistance determinant, *mefA*, from *Streptococcus pyogenes*. Mol Microbiol. 22(5):867-79.

50. Claverys JP, Martin B. 2003. Bacterial "competence" genes: signatures of active transformation, or only remnants? Trends Microbiol. 11(4):161-5.

51. Clewell DB, Flannagan SE, Jaworski DD. 1995. Unconstrained bacterial promiscuity: the Tn*916*-Tn*1545* family of conjugative transposons. Trends Microbiol. 3(6):229-36.

52. Climo MW, Sharma VK, Archer GL. 1996. Identification and characterization of the origin of conjugative transfer (*oriT*) and a gene (*nes*) encoding a single-stranded endonuclease on the staphylococcal plasmid pGO1. J Bacteriol. 178(16):4975-83.

53. Cohen SN, Shapiro JA. 1980. Transposable genetic elements. Sci Am. 242(2):40-9.

54. Collis CM, Hall RM. 1992. Site-specific deletion and rearrangement of integron insert genes catalyzed by the integron DNA integrase. J Bacteriol. 174(5):1574-85.

55. Colquhoun J, Weetch RS. 1950. Resistance to chloramphenicol developing during treatment of typhoid fever. Lancet ii, 621-623.

56. Cooper MG. 2000. The cell: a molecular approach. Chapter 2, the flow of genetic information. Seconde edition. Boston University. ISBN 0-87893-106-6.

57. Costerton JW, Stewart PS, Greenberg EP. 1999. Bacterial biofilms: a common cause of persistent infections. Science. 284(5418):1318-22.

58. Crellin PK, JI Rood. 1998. Tn*4451* from *Clostridium perfringens* is a mobilizable transposon that encodes the functional Mob protein, TnpZ. Molecular Microb. 27(3):631.

59. Cremieux AC, Muller-Serieys C, Panhard X, Delatour F, Tchimichkian M, Mentre F, Andremont A. 2003. Emergence of resistance in normal human aerobic commensal flora during telithromycin and amoxicillin-clavulanic acid treatments. Antimicrob Agents Chemother. 47(6):2030-5.

60. Cresti S, Lattanzi M, Zanchi A, Montagnani F, Pollini S, Cellesi C, Rossolini GM. 2002. Resistance determinants and clonal diversity in group A *streptococci* collected during a period of increasing macrolide resistance. Antimicrob. Agents Chemother. 46:1816-1822.

61. Culebras E, Rodriguez-Avial I, Betriu C, Redondo M, Picazo JJ. 2002. Macrolide and tetracycline resistance and molecular relationships of clinical strains of *Streptococcus agalactiae*. Antimicrob Agents Chemother. 46(5):1574-6.

62. Damrosch DS. 1946. Chemoprophylaxis and sulfonamide resistant streptococci. JAMA 130:124-128.

63. Davies J, Wright GD. 1997. Bacterial resistance to aminoglycoside antibiotics. Trends Microbiol. 5(6):234-40.

64. Davies J. 1994. Inactivation of antibiotics and the dissemination of resistance genes. Science. 264(5157):375-82.

65. Davison J. 1999. Genetic exchange between bacteria in the environment. Plasmid. 42(2):73-91.

66. Debelian GJ, Olsen I, Tronstad L. 1994. Systemic diseases caused by oral microorganisms. End Dent Trauml. 10:57-65.

67. Delahay RM, Robertson BD, Balthazar JT, Shafer WM, Ison CA. 1997. Involvement of the gonococcal MtrE protein in the resistance of *Neisseria gonorrhoeae* to toxic hydrophobic agents. Microbiol. 143:2127–2133

68. Del Grosso M, Scotto d'Abusco A, Iannelli F, Pozzi G, Pantosti A. 2004. Tn*2009*, a Tn*916*-like element containing *mef*(E) in *Streptococcus pneumoniae*. Antimicrob Agents Chemother. 48(6):2037-42.

69. DeLisle S, Perl TM. 2003. Vancomycin-resistant enterococci: a road map on how to prevent the emergence and transmission of antimicrobial resistance. Chest. 123(5 Suppl):504S-18S.

70. Demaneche S, Kay E, Gourbiere F, Simonet P. 2001. Natural transformation of *Pseudomonas fluorescens* and *Agrobacterium tumefaciens* in soil. Appl Environ Microbiol. 67(6):2617-21.

71. De Vries J, Heine M, Harms K, Wackernagel W. 2003. Spread of recombinant DNA by roots and pollen of transgenic potato plants, identified by highly specific biomonitoring using natural transformation of an *Acinetobacter sp.* Appl Environ Microbiol. 69(8):4455-62.

72. Diaz-Torres ML, McNab R, Spratt DA, Villedieu A, Hunt N, Wilson M, Mullany P. 2003. Novel tetracycline resistance determinant from the oral Metagenome. Antimicrob. Agents Chemother. 47:1430-1432.

73. Doern GV, Heilmann KP, Huynh HK, Rhomberg PR, Coffman SL, Brueggemann AB. 2001. Antimicrobial resistance among clinical isolates of *Streptococcus pneumoniae* in the United States during 1999-2000, including a

comparison of resistance rates since 1994-1995. Antimicrob Agents Chemother. 45(6):1721-9.

74. Doern GV, Ferraro MJ, Brueggemann AB, Ruoff KL. 1996. Emergence of high rates of antimicrobial resistance among viridans group streptococci in the United States. Antimicrob. Agents Chemother. 40: 891-894.

75. Domagk G. 1935. A contribution to the chemotherapy of bacterial infections. Dtsch. med. Wochenschr. 61: 250-253. In Milestones in Microbiology: 1556 to 1940, translated and edited by Thomas D. Brock, ASM Press. 1998, p195.

76. Dubnau D. 1999. DNA uptake in bacteria. Annu. Rev. Microbiol. 53:217–44.

77. Dubnau D. 1997. Binding and transport of transforming DNA by *Bacillus subtilis*: the role of type-IV pilin-like proteins--a review. Gene. 192(1):191-8.

78. Dubnau D. 1991. Genetic competence in *Bacillus subtilis*. Microbiol Rev. 55(3):395-424.

79. Edlund C, Alván G, Barkholt L, Vacheron F, Nord CE. 2000. Pharmacokinetics and comparative effects of telithromycin (HMR 3647) and clarithromycin on the oropharyngeal and intestinal microflora. J Antim Chem. 46:741-749.

80. Endtz HP, Ruijs GJ, van Klingeren B, Jansen WH, van der Reyden T, Mouton RP. 1991. Quinolone resistance in campylobacter isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. J Antimicrob Chemother. 27(2):199-208.

81. Enne VI, Livermore DM, Stephens P, Hall LM. 2001. Persistence of sulphonamide resistance in *Escherichia coli* in the UK despite national prescribing restriction. Lancet. 357(9265):1325-8.

82. Farrell DJ, Morrissey I, De Rubeis D, Robbins M, Felmingham D. 2003. A UK multicentre study of the antimicrobial susceptibility of bacterial pathogens causing urinary tract infection. J Infect. 46(2):94-100.

83. Farrell DJ, Morrissey I, Bakker S, Felmingham D. 2001. Detection of macrolide resistance mechanisms in *Streptococcus pneumoniae* and *Streptococcus*

pyogenes using a multiplex rapid cycle PCR with microwell-format probe hybridization. J Antimicrob Chemother. 48(4):541-4.

84. Fenoll A, Jado I, Vicioso D, Perez A, Casal J. 1998. Evolution of *Streptococcus pneumoniae* serotypes and antibiotic resistance in Spain: update (1990 to 1996). J Clin Microbiol. 36(12):3447-54.

85. Feres M, Haffajee AD, Allard K, Som S, Goodson JM, Socransky SS. 2002. Antibiotic resistance of subgingival species during and after antibiotic therapy. J Clin Periodontol. 29(8):724-35.

86. Fine DH, Hammond BF, Loesche WJ. 1998. Clinical use of antibiotics in dental practice. Int J Antimicrob Agents. 9(4):235-8.

87. Firth NK, Ippen-Ihler, Skurray R. 1996. Structure and function of the F factor and mechanism of conjugation. In F. Neidhardt et al. (eds), *Escherichia coli* and Salmonella: Cellular and Molecular Biology, second edition, p. 2377-2401. ASM Press, Washington, D.C.

88. Flannagan SE, Zitzow LA, Su YA, Clewell DB. 1994. Nucleotide sequence of the 18-kb conjugative transposon Tn*916* from *Enterococcus faecalis*. Plasmid. 32(3):350-4.

89. Fleming A. 1929. On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae. Reprinted with permission from *British Journal of Experimental Pathology* 10:226–236. (Now *International Journal of Experimental Pathology.*) Copyright © 1929. Blackwell Science Ltd.

90. Florez C, Garcia-Lopez JL, Martin-Mazuelos E. 1997. Susceptibilities of 55 strains of *Neisseria meningitidis* isolated in Spain in 1993 and 1994. Chemotherapy. 43(3):168-70.

91. Fluit AD, Visser MR, Schmitz FJ. 2001. Molecular detection of antimicrobial resistance. Clin Microbiol Rev. 14(4):836-871.

92. Fraise AP. 2002. Biocide abuse and antimicrobial resistance--a cause for concern? J Antimicrob Chemother. 49(1):11-2.

93. Francois B, Charles M, Courvalin P. 1997. Conjugation transfer of *tet*(S) between strains of *Enterococcus faecalis* is associated with the exchange of large fragments of chromosomal DNA. Microbiology 143:2145-2154.

94. Friedland I, Stinson L, Ikaiddi M, Harm S, Woods GL. 2003. Phenotypic antimicrobial resistance patterns in *Pseudomonas aeruginosa* and *Acinetobacter*: results of a Multicenter Intensive Care Unit Surveillance Study, 1995-2000. Diagnostic Microbiol and Infect Dis. 45:245-250.

95. Fridkin SK, Gaynes RP. 1999. Antimicrobial resistance in intensive care units. Clin Chest Med. 2:303-316.

96. Gawron-Burke C, Clewell DB. 1984. Regeneration of insertionally inactivated streptococcal DNA fragments after excision of transposon Tn*916* in *Escherichia coli*: strategy for targeting and cloning of genes from Gram-positive bacteria. J Bacteriol. 159:214-221.

97. Gay K, Stephens DS. 2001. Structure and dissemination of a chromosomal insertion element encoding macrolide efflux in *Streptococcus pneumoniae*. J Infect Dis. 184(1):56-65.

98. Gay K, Baughman W, Miller Y, Jackson D, Whitney CG, Schuchat A, Farley MM, Tenover F, Stephens DS. 2000. The emergence of *Streptococcus pneumoniae* resistant to macrolide antimicrobial agents: a 6-year population-based assessment. J Infect Dis. 182(5):1417-24.

99. Gerrits MM, Berning M, Van Vliet AH, Kuipers EJ, Kusters JG. 2003. Effects of 16S rRNA gene mutations on tetracycline resistance in *Helicobacter pylori*. Antimicrob Agents Chemother. 47(9):2984-6.

100. Gholizadeh Y, Courvalin P. 2000. Acquired and intrinsic glycopeptide resistance in enterococci. Int J of Antimicrob Agents. 16:S11-S17.

101. Giovanetti E, Brenciani A, Lupidi R, Roberts MC, Varaldo PE. 2003. Presence of the *tet*(O) gene in erythromycin- and tetracycline-resistant strains of *Streptococcus pyogenes* and linkage with either the *mef*(A) or the *erm*(A) gene. Antimicrob Agents Chemother. 47(9):2844-9.

102. Giovanetti E, Montanari MP, Mingoia M, and Varaldo PE. 1999. Phenotypes and genotypes of erythromycin-resistant *Streptococcus pyogenes* strains in Italy and heterogeneity of inducibly resistant strains. Antimicrob Agents Chemother. 43:1935-1940.

103. Girons IS, Bourhy P, Ottone C, Picardeau M, Yelton D, Hendrix RW, Glaser P, Charon N. 2000. The LE1 bacteriophage replicates as a plasmid within *Leptospira biflexa*: construction of an *L. biflexa-Escherichia coli* shuttle vector. J Bacteriol. 182(20):5700-5.

104. Glynn MK, Bopp C, Dewitt W, Dabney P, Mokhtar M, Angulo FJ. 1998. Emergence of multidrug-resistant *Salmonella enterica* serotype typhimurium DT104 infections in the United States. N Engl J Med. 338(19):1333-8.

105. Gonzalez I, Georgiou M, Alcaide F, Balas D, Linares J, de la Campa AG. 1998. Fluoroquinolone resistance mutations in the *parC*, *parE*, and *gyrA* genes of clinical isolates of viridans group streptococci. Antimicrob Agents Chemother. 42(11):2792-8.

106. Goossens H, Sprenger MJ. 1998. Community acquired infections and bacterial resistance. BMJ. 317(7159):654-7.

107. Grandey M, Gorton R, O'Hora A, Hollyoak V. 2004. Communicable diseases in the North East of England. North East Public Health Observatory. ISBN I-903945-26-7.

108. Grohmann E, Muth G, Espinosa M. 2003. Conjugative plasmid transfer in Gram-positive bacteria. Microbiol and Mol Biol Rev. 67(2):277-301.

109. Hanberger H, Garcia-Rodriguez JA, Gobernado M, Goossens H, Nilsson LE, Struelens MJ. 1999. Antibiotic susceptibility among aerobic Gram-negative

bacilli in intensive care units in 5 European countries. French and Portuguese ICU Study Groups. JAMA. 281(1):67-71.

110. Hagman KE, Lucas CE, Balthazar JT, Snyder L, Nilles M, Judd RC, Shafer WM. 1997. The MtrD protein of *Neisseria gonorrhoeae* is a member of the resistance/nodulation/division protein family constituting part of an efflux systerm. Microbiol. 143:2117-2125.

111. Hartley DL, Jones KR, Tobian JA, LeBlanc DJ, Macrina FL. 1984. Disseminated tetracycline resistance in oral streptococci: implication of a conjugative transposon. Infection Immunity 45:13.

112. Havarstein LS, Hakenbeck R, Gaustad P. 1997. Natural competence in the genus *Streptococcus*: evidence that streptococci can change pherotype by interspecies recombinational exchanges. J Bacteriol. 179(21):6589-94.

113. Hawley RJ, Lee LN, LeBlanc DJ. 1980. Effects of tetracycline on the streptococcal flora of periodontal pockets. Antimicrob Agents Chemother. 17(3):372-8.

114. Heaton MP, Discotto LF, Pucci MJ, Handwerger S. 1996. Mobilization of vancomycin resistance by transposon-mediated fusion of a VanA plasmid with an *Enterococcus faecium* sex pheromone-response plasmid. Gene 171(1):9-17.

115. Hendrix RW. 2002. Bacteriophages: evolution of the majority. Theor Popul Biol. 61(4):471-80.

116. Herrera D, van Winkelhoff AJ, Dellemijn-Kippuw N, Winkel EG, Sanz M. 2000. Beta-lactamase producing bacteria in the subgingival microflora of adult patients with periodontitis. A comparison between Spain and The Netherlands. J Clin Periodontol. 27(7):520-5.

117. Hiramatsu K, Aritaka N, Hanaki H, Kawasaki S, Hosoda Y, Hori S, Fukuchi Y, Kobayashi I. 1997. Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. Lancet 350:1670-1673.

118. Hiramatsu K, Hanaki H, Ino T, Yabuta K, Oguri T, Tenover FC. 1997. Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. J Antimicrob Chemother. 40(1):135-6.

119. Hitch G, Pratten J, Taylor PW. 2004. Isolation of bacteriophages from the oral cavity. Lett App Microbiol. 39:215-219.

120. Hoiby N. 2000. Ecological antibiotic policy. J Antim Chem. 46 (S1):59-62.

121. Hooton TM, Levy SB. 2001. Antimicrobial resistance: a plan of action for community practice. Am Fam Physician. 63(6):1087-98.

122. House of Lords Report. Session 1997-98. Science and Technology - Seventh Report.

123. Hughes VM, Datta N. 1983. Conjugative plasmids in bacteria of the 'pre-antibiotic' era. Nature 302(5910):725-6.

124. Hwa Lee J. 2003. Methicillin (oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. App Env Microbial. 69(11):6489-6494.

125. Ingman T, Sorsa T, Suomalainen K, Halinen S, Lindy O, Lauhio A, Saari H, Konttinen YT, Golub LM. 1993. Tetracycline inhibition and the cellular source of collagenase in gingival crevicular fluid in different periodontal diseases. A review article. J Periodontol. 1993 Feb;64(2):82-8.

126. Ioannidou S, Tassios PT, Kotsovili-Tseleni A, Foustoukou M, Legakis NJ, Vatopoulos A. 2001. Antibiotic resistance rates and macrolide resistance phenotypes of viridans group streptococci from the oropharynx of healthy Greek children. Int J Antimicrob Agents. 17(3):195-201.

127. Jacobs JA, van Baar GJ, London NH, Tjhie JH, Schouls LM, Stobberingh EE. 2001. Prevalence of macrolide resistance genes in clinical isolates of the *Streptococcus anginosus* ("*S. milleri*") group. Antimicrob Agents Chemother. 45:2375-2377.

128. Janoir C, Podglajen I, Kitzis MD, Poyart C, Gutmann L. 1999. In vitro exchange of fluoroquinolone resistance determinants between *Streptococcus pneumoniae* and viridans streptococci and genomic organization of the parE-parC region in S. mitis. J Infect Dis. 180(2):555-8.

129. Jensen LB, Willems RJL, Van Den Bogaard AE. 2003. Genetic characterization of glycopeptide-resistant enterococci of human and animal origin from mixed pig and poultry farms. APMIS 111: 669–72.

130. Jensen LB, Ahrens P, Dons L, Jones RN, Hammerum AM, Aarestrup FM. 1998. Molecular analysis of Tn*1546* in *Enterococcus faecium* isolated from animals and humans. J Clin Microbiol. 36(2):437-42.

131. Jiang SC, Paul JH. 1998. Gene transfer by transduction in the marine environment. Appl Environ Microbiol. 64(8):2780-7.

132. Johnston AM. 1998. Use of antimicrobial drugs in veterinary practice. BMJ. 1998 317(7159):665-7.

133. Kaatz GW, Seo SM. 1999. Mechanisms of fluoroquinolone resistance in genetically related strains of *Staphylococcus aureus*. Antimicrob Agents Chemother. 41(12):2733-7.

134. Kataja J, Huovinen P, Skurnik M, the Finnish Study Group for Antimicrobial Resistance and Seppälä H. 1999. Erythromycin resistance genes in group A Streptococci in Finland. Antimicrob Agents Chemother. 43:48-52.

135. Katayama Y, Ito T, Hiramatsu K. 2000. A new class of genetic element, staphylococcal cassette chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. 44:1549-1555.

136. Kehrenberg C, Salmon SA, Watts JL, Schwarz S. 2001. Tetracycline resistance genes in isolates of *Pasteurella multocida*, *Mannheimia haemolytica*, *Mannheimia glucosida* and *Mannheimia varigena* from bovine and swine respiratory disease: intergeneric spread of the *tet*(H) plasmid pMHT1. J Antimicrob Chemother 48(5):631-40.

137. Kiple KF. 1993. Cambridge World History of Human Disease. Cambridge University Press, Cambridge, New York.

138. Kirst HA. 1991. New macrolides: expanded horizons for an old class of antibiotics. J Antimicrob Chemother. 1991 Dec;28(6):787-90.

139. Kjeldgaard M, Nyborg J. 1992. Refined structure of elongation factor EF-Tu from *Escherichia coli*. J Mol Biol. 223(3):721-42.

140. Koh J, Wilson M, Vidic J, Newman HN. 1986. Amoxycillin-resistant streptococci in dental plaque. Microbios. 45(182):41-53.

141. Köhler T, Pechere JC, Plesiat P. 1999. Bacterial antibiotic efflux systems of medical importance. Cell Mol Life Sci. 56(9-10):771-8.

142. Lacroix JM, Walker CB. 1996. Detection and prevalence of the tetracycline resistance determinant Tet Q in the microbiota associated with adult periodontitis. Oral Microbiol Immunol. 11:282-288.

143. Lacroix JM, Walker CB. 1995. Detection and incidence of the tetracycline resistance determinant *tet*(M) in the microflora associated with adult periodontitis. J Periodontol. 66:102-108.

144. Lancaster H, Roberts AP, Bedi R, Wilson M, Mullany P. 2004. Characterization of Tn*916S*, a Tn*916*-like element containing the tetracycline resistance determinant tet(S). J Bacteriol. 186(13):4395-8.

145. Lane DJ. 1996. 16S/23S rRNA sequencing, p. 115-175. *In* E. Stackebrandt, and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. Wiley and Sons Ltd., Chichester, United Kingdom.

146. Lawrence JG, Ochman H. 1997. Amelioration of bacterial genomes: rates of change and exchange. J Mol Evol. 44(4):383-97.

147. Le Bouguenec C, de Cespedes G, Horaud T. 1990. Presence of chromosomal elements resembling the composite structure Tn*3701* in streptococci. J Bacteriol. 172(2):727-34.

148. Leclercq R. 2002. Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. Clin Infect Dis. 34(4):482-92.

149. Leclercq R, Derlot E, Duval J, Courvalin P. 1987. Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. N Engl J Med. 319:157-61.

150. Legg JA, Wilson M. 1990. The prevalence of beta-lactamase producing bacteria in subgingival plaque and their sensitivity to Augmentin. Br J Oral Maxillofac Surg. 28(3):180-4.

151. Leng Z, Riley DE, Berger DE, Kneger JN, Roberts MC. 1997. Distribution and mobility of the tetracycline resistance determinant Tet Q. J Antimicrob Chemoter. 40:551-559.

152. Letellier L, Plancon L, Bonhivers M, Boulanger P. 1999. Phage DNA transport across membranes. Res Microbiol. 150(8):499-505.

153. Levy SB. 2001. Antibacterial household products: cause for concern. Emerg Infect Dis. 7(3 Suppl):512-5.

154. Levy SB, McMurry LM, Barbosa TM, Burdett V, Courvalin P, Hillen W, Roberts MC, Rood JI, Taylor DE. 1999. Nomenclature for new tetracycline resistance determinants. Antimicrob Agents Chemother. 43(6):1523-4.

155. Levy SB. 1998. "The Challenge of Antibiotic Resistance." *Scientific American.*

156. Levy SB. 1992. Active efflux mechanisms for antimicrobial resistance. Antimicrob Agents Chemother. 36:695-703.

157. Lewis K. 1994. Multidrug resistance pumps in bacteria: variations on a theme. Trends Biochem Sci. 19:119-23.

158. Li YH, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG. 2001. Natural genetic transformation of *Streptococcus mutans* growing in biofilms. J Bacteriol. 183(3):897-908.

159. Li LY, Shoemaker NB, Salyers AA. 1993. Characterization of the mobilization region of a *Bacteroides* insertion element (NBU1) that is excised and transferred by Bacteroides conjugative transposons. J Bacteriol. 175(20):6588-98.

160. Licht TR, Christensen BB, Krogfelt KA, Molin S. 1999. Plasmid transfer in the animal intestine and other dynamic bacterial populations: the role of community structure and environment. Microbiology. 145:2615-22.

161. Livermore DM. 2000. Antibiotic resistance in staphylococci. International Journal of Antimicrobial Agents. 16:S3–S10.

162. Loesche W. 1997. Association of the oral flora with important medical diseases. Curr Opin Period. 4:21-28.

163. Lorenz MG, Wackernagel W. 1994. Bacterial gene transfer by natural genetic transformation in the environment. Microbiol Rev. 58(3):563-602.

164. Luna VA, Heiken M, Judge K, Ulep C, Van Kirk N, Luis H, Bernardo M, Leitao J, Roberts MC. 2002. Distribution of *mef*(A) in Gram-positive bacteria from healthy portuguese children. Antimicrob Agents Chemother. 46:2513-2517.

165. Luna VA, Coates P, Eady EA, Cove JH, Nguyen TT, Roberts MC. 1999. A variety of Gram-positive bacteria carry mobile *mef* genes. J Antimicrob Chemother. 44:19-25.

166. Luo N, Sahin O, Lin J, Michel LO, Zhang Q. 2003. In vivo selection of Campylobacter isolates with high levels of fluoroquinolone resistance associated with *gyrA* mutations and the function of the CmeABC efflux pump. Antimicrob Agents Chemother. 47(1):390-4.

167. MacGowan AP, Wise R. 2001. Establishing MIC breakpoints and the interpretation of in vitro susceptibility tests. J Antimicrob Chemother. 48 Suppl 1:17-28.

168. Mah TF, O'Toole GA. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 9(1):34-9.

169. Mahillon J, Chandler M. 1998. Insertion sequences. Microbiol Mol Biol Rev. 62(3):725-74.

170. Marimon JM, Gomariz M, Zigorraga C, Cilla G, Perez-Trallero E. 2004. Increasing prevalence of quinolone resistance in human nontyphoid Salmonella enterica isolates obtained in Spain from 1981 to 2003. Antimicrob Agents Chemother. 2004 Oct;48(10):3789-93.

171. Marshall CG, Lessard IAD, Park IS, Wright GD. 1998. Glycopeptide antibiotic resistance genes in glycopeptide-producing organisms. Antimicrob. Agents Chemoter. 42(9):2215-2220.

172. Marshall CG, Broadhead G, Leskiw BK, Wright GD. 1997. D-Ala-D-Ala ligases from glycopeptide antibiotic-producing organisms are highly homologous to the enterococcal vancomycin-resistance ligases VanA and VanB. Proc Natl Acad Sci USA. 94(12):6480-3.

173. Martin JM, Green M, Barbadora KA, Wald ER. 2002. Erythromycin-resistant group A streptococci in schoolchildren in Pittsburgh. N Engl J Med. 346(16):1200-6.

174. McDougal LK, Tenover FC, Lee LN, Rasheed JK, Patterson JE, Jorgensen JH, LeBlanc DJ. 1998. Detection of Tn*917*-like sequences within a Tn*916*-like conjugative transposon (Tn*3872*) in erythromycin-resistant isolates of *Streptococcus pneumoniae*. Antimicrob Agents Chemother. 42(9):2312-8.

175. McGee L, Klugman KP, Wasas A, Capper T, Brink A, and The Antibiotics Surveillance Forum Of South Africa. 2001. Serotype 19F multiresistant pneumococcal clone harboring two erythromycin resistance determinants [*erm*(B) and *mef*(A)] in South Africa. Antimicrob Agents Chemother. 45:1595-1598.

176. McManus PS, Stockwell VO, Sundin GW, Jones AL. 2002. Antibiotic use in plant agriculture. Annu Rev Phytopathol. 40:443-65.

177. McMurry LM, Oethinger M, Levy SB. 1998. Overexpression of *marA*, *soxS*, or *acrAB* produces resistance to triclosan in laboratory and clinical strains of *Escherichia coli*. FEMS Microbiol Lett. 166(2):305-9.

178. Medeiros AA. 1997. Evolution and dissemination of β-lactamases accelerated by generations of β-lactam antibiotics. Clin Inf Dis. 24(Suppl 1):S19-45.

179. Melville CM, Brunel R, Flint HJ, Scott KP. 2004. The *Butyrivibrio fibrisolvens tet*(W) gene is carried on the novel conjugative transposon Tn*B1230*, which contains duplicated nitroreductase coding sequences. J Bacteriol. 186(11):3656-9.

180. Melville CM, Scott KP, Mercer DK, Flint HJ. 2001. Novel tetracycline resistance gene, *tet*(32), in the Clostridium-related human colonic anaerobe K10 and its transmission in vitro to the human anaerobe *Butyrivibrio fibrisolvens*. Antimicrob Agents Chemoter. 45(11):3242-3249.

181. Mercer DK, Scott KP, Melville CM, Glover LA, Flint HJ. 2001. Transformation of an oral bacterium via chromosomal integration of free DNA in the presence of human saliva. FEMS Microbiol Lett. 200(2):163-7.

182. Mercer DK, Scott KP, Bruce-Johnson WA, Glover LA, Flint HJ. 1999. Fate of free DNA and transformation of the oral bacterium *Streptococcus gordonii* DL1 by plasmid DNA in human saliva. Appl Environ Microbiol. 65(1):6-10.

183. Miller RV, Day MJ (ed.). 2004. Microbial evolution, gene establishment, survival, and exchange. ASM press, Washington, D.C.

184. Molin S, Tolker-Nielsen T. 2003. Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. Curr Opin Biotechnol. 14(3):255-61.

185. Montanari MP, Cochetti I, Mingoia M, Varaldo PE. 2003. Phenotypic and molecular characterization of tetracycline- and erythromycin-resistant strains of *Streptococcus pneumoniae*. Antimicrob Agents Chemother. 47(7):2236-41.

186. Mörner KAH. 1967. Nobel Lectures, Physiology or Medicine 1901-1921, Elsevier Publishing Company, Amsterdam, The Nobel Prize in Physiology or Medicine 1905. Presentation Speech by Professor the Count K.A.H. Mörner, Rector of the Royal Caroline Institute.

187. Morse SA, Lysko PG, McFarland L, Knapp JS, Sandstrom E, Critchlow C, Holmes KK. 1982. Gonococcal strains from homosexual men have outer membranes with reduced permeability to hydrophobic molecules. Inf and Imm. 37(2):432-438.

188. Mullany P, Roberts AP, Wang H. 2002. Mechanism of integration and excision in conjugative transposons. Cell Mol Life Sci. 59:2017-2022.

189. Mullany P. 2000. Gene transfer in the GI tract and the oral cavity. Microbial Ecology in Health and Disease. Suppl 2:73-80.

190. Murdoch DA. 1998. Gram-positive anaerobic cocci. Clin Microbiol Rev. 11(1):81-120.

191. Nagai K, Shibasaki Y, Hasegawa K, Davies TA, Jacobs MT, Ubukata K, Appelbaum PC. 2001. Evaluation of PCR primers to screen for *Streptococcus pneumoniae* isolates and ß-lactam resistance, and to detect common macrolide resistance determinants. J Antimicrob Chemother. 48:915-918.

192. National Committee for Clinical Laboratory Standards. 1993. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 3rd ed. Approved standard M7-A3. National Committee for Clinical Labo-ratory Standards, Wayne, Pa.

193. Nawaz MS, Khan SA, Khan AA, Khambaty FM, Cerniglia CE. 2000. Comparative molecular analysis of erythromycin-resistance determinants in staphylococcal isolates of poultry and human origin. Mol Cell Probes. 14:311-319.

194. Ng L-K, Martin I, Liu G, Bryden L. 2002. Mutation in 23S rRNA associated with macrolide resistance in *Neisseria gonorrhoeae*. Antimicrob Agents Chemother. 46(9):3020-5.

195. Ng L-K, Martin I, Alfo M, Mulvey M. 2001. Multiplex PCR for the detection of tetracycline resistance genes. Molecular and Cellular Probes 15:209-215.

196. Ng L-K, Stiles ME, Taylor DE. 1987. DNA probes for identification of tetracycline resistance genes in *Campylobacter* species isolated from swine and cattle. Antimicrob Agents Chemother. 31(11):1669-74.

197. Nield BS, Holmes AJ, Gillings MR, Recchia GD, Mabbutt BC, Nevalainen KM, Stokes HW. 2001. Recovery of new integron classes from environmental DNA. FEMS Microbiol Lett. 195(1):59-65.

198. Nielsen KM, Smalla K, van Elsas JD. 2000. Natural transformation of *Acinetobacter* sp. strain BD413 with cell lysates of *Acinetobacter* sp., *Pseudomonas fluorescens*, and *Burkholderia cepacia* in soil microcosms. Appl Environ Microbiol. 66(1):206-12.

199. Nikolich NP, Schoemaker NB, Salyers AA. 1992. A *Bacteroides* tetracycline resistance gene represents a new class of ribosomal protective tetracycline resistance. Antimicrob. Agents Chemoter. 36(5):1005-1012.

200. NNIS System. 2003. National Nosocomial Infections Surveillance (NNIS) System Report, data summary from January 1992 through June 2003, issued August 2003. Am J Infect Control. 31:481-98.

201. NNIS System. 2001. National Nosocomial Infections Surveillance (NNIS) System Report, Data Summary from January 1992-June 2002, Issued August 2002. Am J Infect Control. 30:458-75.

202. NNIS System. 2000. National Nosocomial Infections Surveillance (NNIS) System Report, Data Summary from January 1992-June 2001, Issued August 2001. Am J Infect Control. 29:404-21.

203. Noguchi N, Katayama J, Sasatsu M. 2000. A transposon carrying the gene *mph*(B) for macrolide 2'-phosphotransferase II. FEMS Microbiol Lett. 192(2):175-8.

204. Noguchi N, Tamura Y, Katayama J, Narui K. 1998. Expression of the *mph*(B) gene for macrolide 2'-phosphotransferase II from *Escherichia coli* in *Staphylococcus aureus*. FEMS Microbiol Lett. 159(2):337-42.

205. Noguchi N, Emura A, Matsuyama H, O'Hara K, Sasatsu M, Kono M. 1995. Nucleotide sequence and characterization of erythromycin resistance determinant that encodes macrolide 2'-phosphotransferase I in *Escherichia coli*. Antimicrob Agents Chemother. 39(10):2359-63.

206. Nonaka L, Suzuki S. 2002. New Mg^{2+}-dependent oxytetracycline resistance determinant Tet 34 in *Vibrio* isolates from marine fish intestinal contents. Antimicrob Agents Chemoter. 46(5):1550-1552.

207. Norgren M, Scott JR. 1991. The presence of conjugative transposon Tn*916* in the recipient strain does not impede transfer of a second copy of the element. J Bacteriol. 173(1):319-24.

208. Ochman HJ, Wilson AC. 1987. Evolution in bacteria: evidence for a universal substitution rate in cellular genomes. J Mol Evol. 26:74-86.

209. Olsvik B, Hansen BF, Tenover FC, Olsen I. 1995. Tetracycline-resistant micro-organisms recovered from patients with refractory periodontal disease. J Clin Periodontol. 22(5):391-6.

210. Olsvik B, Olsen I, Tenover FC. 1995. Detection of *tet*(M) and *tet*(O) using the polymerase chain reaction in bacteria isolated from patients with periodontal disease. Oral Microbiol Immunol. 10:87-92.

211. Olsvik B, Olsen I, Tenover FC. 1994. The *tet*(Q) gene in bacteria isolated from patients with refractory periodontal disease. Oral. Microbiol. Immunol. 10:87-92.

212. Osborn AM, Böltner D. 2002. When phages, plasmids and transposons collide: genomic islands and conjugative- and mobilizable-transposons as a mosaic continuum. Plasmid. 48:202 –212.

213. Palmer EL, Teviotdale BL, Jones AL. 1997. A relative of the broad-host-range plasmid RSF1010 detected in *Erwinia amylovora*. Appl Environ Microbiol. 63(11):4604-7.

214. Pancoast SJ. 1988. Aminoglycoside antibiotics in clinical use. Med. Clin. North. Am. 72:581-602.

215. Partridge SR, Recchia GD, Scaramuzzi C, Collis CM, Stokes HW, Hall RM. 2000. Definition of the *attI1* site of class 1 integrons. Microbiology. 146:2855–2864.

216. Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A, Dewhirst FE. 2001. Bacterial diversity in human subgingival plaque. J Bacteriol. 183(12):3770-83.

217. Paulsen IT, Brown MH, Skurray RA. 1996. Proton-dependent multidrug efflux systems. Microbiol Rev. 60(4):575-608.

218. Pérez-Trallero E, Vicente D, Montes M, Marimon JM, and Piñeiro L. 2001. High proportion of pharyngeal carriers of commensal streptococci resistant to erythromycin in Spanish adults. J. Antimicrob. Chemother. 48: 225-229.

219. Pérez-Trallero E, Arenzana JMG, Ayestaran I, Muňoz Baroja I. 1989. Comparative activity in vitro of 16 antimicrobial agents against penicillin-susceptible meningococci and meningococci with diminished susceptibility to penicillin. Antimicrob Agents Chemother. 33(9)1622-1623.

220. Pezzella C, Ricci A, DiGiannatale E, Luzzi I, Carattoli A. 2004. Tetracycline and streptomycin resistance genes, transposons, and plasmids in *Salmonella enterica* isolates from animals in Italy. Antimicrob Agents Chemother. 48(3):903-8.

221. Piddock LJ. 1995. Quinolone resistance and *Campylobacter* spp. J Antimicrob Chemother. 1995 Dec;36(6):891-8.

222. Pike R, Lucas V, Stapleton P, Gilthorpe MS, Roberts G, Rowbury R, Richards H, Mullany P, Wilson M. 2002. Prevalence and antibiotic resistance

profile of mercury-resistant oral bacteria from children with and without mercury amalgam fillings. J Antimicrob Chemother. 49(5):777-83.

223. Poyart C, Pierre C, Quesne G., Pron B, Berche P, Trieu-Cuot P. 1997. Emergence of vancomycin resistance in the genus *Streptococcus*: characterization of a *vanB* transferable determinant in *Streptococcus bovis*. Antimicrob. Agents Chemoter. 41(1):24-29.

224. Pradier C, Dunais B, Carsenti-Etesse H, Dellamonica P. 1997. Pneumococcal resistance patterns in Europe. Eur J Clin Microbiol Infect Dis. 16(9):644-7.

225. Prescott LM, Harley JP, Klein DA. 1996. Microbiology third edition. Chapter 1, the history and scope of microbiology, 1-16.

226. Provvedi R, Dubnau D. 1999. ComEA is a DNA receptor for transformation of competent Bacillus subtilis. Mol Microbiol. 31(1):271-80.

227. Public Health Laboratory Services. 2003. World TB day-24 March 2003. CDR weekly 20 March 2003.

228. Rasmussen JL, Odelson DA, Macrina FL. 1987. Complete nucleotide sequence of insertion element IS*4351* from *Bacteroides fragilis*. J Bacteriol. 169(8):3573-80.

229. Reacher MH, Shah A, Livermore DM, Wale MC, Graham C, Johnson AP, Heine H, Monnickendam MA, Barker KF, James D, George RC. 2000. Bacteraemia and antibiotic resistance of its pathogens reported in England and Wales between 1990 and 1998: trend analysis. BMJ. 320(7229):213-6.

230. Ready D, Roberts AP, Pratten J, Spratt DA, Wilson M, Mullany P. 2002. Composition and antibiotic resistance profile of microcosm dental plaques before and after exposure to tetracycline. J Antimicrob Chemother. 49(5):769-75.

231. Recchia GD, Hall RM. 1997. Origins of the mobile gene cassettes found in integrons. Trends Microbiol. 5(10):389-94.

232. Reichmann P, Konig A, Linares J, Alcaide F, Tenover FC, McDougal L, Swidsinski S, Hakenbeck R. 1997. A global gene pool for high-level

cephalosporin resistance in commensal *Streptococcus* species and *Streptococcus pneumoniae*. J Infect Dis. 176(4):1001-12.

233. Reig M, Galan J, Baquero F, Perez-Diaz JC. 2001. Macrolide resistance in *Peptostreptococcus* spp. mediated by *ermTR*: possible source of macrolide-lincosamide-streptogramin B resistance in *Streptococcus pyogenes*. Antimicrob Agents Chemother. 45:630-632.

234. Reinert RR, Al-Lahham A, Lemperle M, Tenholte C, Briefs C, Haupts S, Gerards HH, Lutticken R. 2002. Emergence of macrolide and penicillin resistance among invasive pneumococcal isolates in Germany. J Antimicrob Chemother. 49(1):61-8.

235. Reynolds PE. 1989. Structure, biochemistry and mechanism of action of glycopeptide antibiotics. Eur J Clin Microbiol Infect Dis. 8(11):943-50.

236. Rice LB. 2002. Association of different mobile elements to generate novel integrative elements. Cell Mol Life Sci. 59(12):2023-32.

237. Rice LB. 1998. Tn*916* family conjugative transposons and dissemination of antimicrobial resistance determinants. Antimicrob Agents Chemoter. 42(8):1871-1877.

238. Ridenhour MB, Fletcher HM, Mortensen JE, Daneo-Moore L. 1996. A novel tetracycline-resistant determinant, *tet*(U), is encoded on the plasmid pKq10 in *Enterococcus faecium*. Plasmid. 35(2):71-80.

239. Robert C, Moellering JR. 1998. Vancomycin-Resistant Enterococci. Clinical Infectious Diseases 26:1196–9.

240. Robert VM, Day MJ. 2004. Microbial evolution, gene establishment, survival and exchange. ASM Press, Washington, DC, p144-157.

241. Roberts AP, Hennequin C, Elmore M, Collignon A, Karjalainen T, Minton N, Mullany P. 2003. Development of an integrative vector for the expression of antisense RNA in *Clostridium difficile*. J Microb Methods. 55:617-24.

242. Roberts AP, Braun V, von Eichel-Streiber C, Mullany P. 2001. Demonstration that the group II intron from the clostridial conjugative transposon Tn*5397* undergoes splicing *in vivo*. J Bacteriology. 183:1296-1299.

243. Roberts AP, Cheah G, Ready D, Pratten J, Wilson M, Mullany P. 2001. Transfer of Tn*916*-like elements in microsm dental plaques. Antimicrob Agents Chemoter. 45(10):2943-2946.

244. Roberts AP, Pratten J, Wilson M, Mullany P. 1999. Transfer of a conjugative transposon, Tn*5397* in a model oral biofilm. FEMS Microbiol Lett. 177(1):63-6.

245. Roberts MC. 2000. Antibiotic toxicity, interactions and resistance development. Periodontol 2000 28:280-297.

246. Roberts MC, Chung WO, Darcie R, Xia M, Marquez C, Borthagaray G, Whittington WL, Holmes KK. 1999. Erythromycin-resistant *Neisseria gonorrhoeae* and oral commensal *Neisseria* spp carry known rRNA methylase genes. Antimicrob Agents Chemother. 43(6):1367-1372.

247. Roberts MC, Sutcliffe J, Courvalin P, Jensen LB, Rood J, Seppala H. 1999. Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. Antimicrob Agents Chemother. 43(12):2823-30.

248. Roberts MC. 1998. Antibiotic resistance in oral/respiratory bacteria. Crit Rev Oral Biol Med. 9:522-540.

249. Roberts MC. 1996. Tetracycline-resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. FEMS Microbiol.Rev. 19:1–24.

250. Rohwer F, Edwards R. 2002. The Phage Proteomic Tree: a genome-based taxonomy for phage. J Bacteriol. 184(16):4529-35.

251. Romanowski G, Lorenz MG, Wackernagel W. 1993. Plasmid DNA in a groundwater aquifer microcosm--adsorption, DNAase resistance and natural genetic transformation of *Bacillus subtilis*. Mol Ecol. 2(3):171-81.

252. Ross JI, Eady EA, Cove JH, Cunliffe WJ. 1998. 16S rRNA mutation associated with tetracycline resistance in a Gram-positive bacterium. Antimicrob Agents Chemother. 42(7):1702-5.

253. Rudy CK, Scott JR. 1994. Length of the coupling sequence of Tn*916*. J Bacteriol. 176(11):3386-8.

254. Salyers AA, Amabile-Cuevas CF. 1997. Why are antibiotic resistance genes so resistant to elimination? Antimicrob Agents Chemother. 41(11):2321-5.

255. Salyers AA, Shoemaker NB, Stevens AM, Li LY. 1995. Conjugative transposons: an unusual and diverse set of integrated gene transfer elements. Microbiol Rev. 59(4):579-590.

256. Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning. A laboratory manual, second edition. Cold Spring Harbour Laboratory Press. USA.

257. Sanchez L, Pan W, Vinas M, Nikaido H. 1997. The *acrAB* homolog of *Haemophilus influenzae* codes for a functional multidrug efflux pump. J Bacteriol. 1997 Nov;179(21):6855-7.

258. Sanger F, Coulson AR, Hong GF, Hill DF, Petersen GB. 1982. Nucleotide sequence of bacteriophage lambda DNA. J Mol Biol. 162(4):729-73.

259. Santagati M, Iannelli F, Cascone C, Campanile F, Oggioni MR, Stefani S, Pozzi G. 2003. The novel conjugative transposon Tn*1207.3* carries the macrolides efflux gene *mef*(A) in *Streptococcus pyogenes*.

260. Santagati M, Iannelli F, Oggioni MR, Stefani S, Pozzi G. 2000. Characterization of a genetic element carrying the macrolide efflux gene *mef*(A) in *Streptococcus pneumoniae*. Antimicrob Agents Chemother. 44(9):2585-7.

261. Schaaff F, Reipert A, Bierbaum G. 2002. An elevated mutation frequency favors development of vancomycin resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother. 46(11):3540-3548.

262. Schalen C, Gebreselassie D, Stahl S. 1995. Characterization of an erythromycin resistance (*erm*) plasmid in *Streptococcus pyogenes*. APMIS. 103(1):59-68.

263. Schnabel EL, Jones AL. 1999. Distribution of tetracycline resistance genes and transposons among phylloplane bacteria in Michigan apple orchards. Appl Environ Microbiol. 65(11):4898-907.

264. Scott KP, Melville CM, Barbosa TM, Flint W. 2000. Occurrence of the new tetracycline resistance gene *tet*(W) in bacteria from the human gut. Antimicrob Agents Chemoter. 44:775-777.

265. Seppäla H, Haanpera M, Al-Juhaish M, Jarvinen H, Jalava J, Huovinen P. 2003. Antimicrobial susceptibility patterns and macrolide resistance genes of viridans group streptococci from normal flora. J Antimicrob Chemother. 52(4):636-44.

266. Seppäla H, Klaukka T, Lehtonen R, Nenonen E, the Finnish Dtudy Group for Antimicrobial Resistance, and Huovinen P. 1995. Outpatient use of erythromycin: link to increased erythromycin resistance in group A streptococci. Clin Inf Dis. 21:1378-1385.

267. Seral C, Castillo FJ, Rubio-Calvo MC, Fenoll A, García C, Gómez-Lus R. 2001. Distribution of resistance genes *tet*(M), aph3'-III, catpC194 and the integrase gene of Tn*1545* in clinical *Streptococcus pneumoniae* harbouring *erm*(B) and *mef*(A) genes in Spain. J Antimicrob Chemother. 47:863-866.

268. Shinzato T, Saito A. 1995. The *Streptococcus milleri* group as a cause of pulmonary infections. Clin Infect Dis. 21 (Suppl 3):S238-243.

269. Shoemaker NB, Vlamakis H, Hayes K, Salyers AA. 2001. Evidence for extensive resistance gene transfer among *Bacteroides* spp and among *Bacteroides* and other genera in the human colon. Appl Environ Microbiol. 67(2):561-568.

270. Sidhu MS, Heir E, Sorum H, Holck A. 2001. Genetic linkage between resistance to quaternary ammonium compounds and beta-lactam antibiotics in food-related *Staphylococcus* spp. Microb Drug Resist. 7(4):363-71.

271. Smith M, Marsh P, Wilson M. 1990. β-Lactamase-producing bacteria in the dental plaque of children. 1990. J Paed Dent. 6:15-21.

272. Solomon JM, Grossman AD. 1996. Who's competent and when: regulation of natural genetic competence in bacteria. Trends Genet. 12: 150-155.

273. Sougakoff W, Papadopoulou B, Nordmann P, Courvalin P. 1987. Nucleotide sequence and distribution of gene *tet*(O) encoding tetracycline resistance in *Campylobacter coli*. FEMS Microbiol Lett. 44:153-159.

274. Speer BS, Shoemaker NB, Salyers AA. 1992. Bacterial resistance to tetracycline: mechanism, transfer, and clinical significance. Clin Microbiol Rev. 5(4):37-399.

275. Speer BS, Bedzyk L, Salyers AA. 1991. Evidence that a novel tetracycline resistance gene found on two bacteroides transposons encodes an NADP-requiring oxidoreductase. J Bacteriol. 173(1):176-83.

276. Spratt BG. 1994. Resistance to antibiotics mediated by target alterations. Science. 1994 Apr 15;264(5157):388-93.

277. Srinivasan A, Dick JD, Perl TM. 2002. Vancomycin resistance in staphylococci. Clinic Microb Rev. 15(3):430-438.

278. Stanton TB, McDowall JS, Rasmussen MA. 2004. Diverse tetracycline resistance genotypes of *Megasphaera elsdenii* strains selectively cultured from swine feces. Appl Environ Microbiol. 70(6):3754-7.

279. Stanton TB, Humphrey SB. 2003. Isolation of tetracycline-resistant *Megasphaera elsdenii* strains with novel mosaic gene combinations of *tet*(O) and *tet*(W) from swine. Appl Environ Microbiol. 69(7):3874-82.

280. Stevens AM, Shoemaker NB, Li LY, Salyers AA. 1993. Tetracycline regulation of genes on Bacteroides conjugative transposons. J Bacteriol. 175(19):6134-41.

281. Stobberingh E, van den Bogaard A, London N, Driessen C, Top J, Willems R. 1999. Enterococci with glycopeptide resistance in turkeys, turkey farmers, turkey slaughterers, and (sub)urban residents in the south of The Netherlands: evidence for transmission of vancomycin resistance from animals to humans? Antimicrob Agents Chemother. 43(9):2215-21.

282. Su YA, He P, Clewell DB. 1992. Characterization of the *tet*(M) determinant of Tn*916*: evidence for regulation by transcription attenuation. Antimicrob. Agents Chemother. 36:769–778.

283. Summers AO, Wireman J, Vimy MJ, Lorscheider FL, Marshall B, Levy SB, Bennett S, Billard L. 1993. Mercury released from dental "silver" fillings provokes an increase in mercury- and antibiotic-resistant bacteria in oral and intestinal floras of primates. Antimicrob Agents Chemother. 1993 Apr;37(4):825-34.

284. Sutcliffe J, Grebe T, Tait-Kamradt A, Wondrack L. 1996. Detection of erythromycin-resistant determinants by PCR. Antimicrob Agents Chemother. 40:2562-2566.

285. Sutcliffe J, Tait-Kamradt A, Wondrack L. 1996. *Streptococcus pneumoniae* and *Streptococcus pyogenes* resistant to macrolides but sensitive to clindamycin: a common resistance pattern mediated by an efflux system. Antimicrob Agents Chemother. 40:1817-1824.

286. Svartz N. 1965. Nobel Lectures, Physiology or Medicine 1922-1941, Elsevier Publishing Company, Amsterdam, The Nobel Prize in Physiology or Medicine 1939. Presentation Speech by Professor N. Svartz, member of the Staff of Professors of the Royal Caroline Institute.

287. Swann Committee, her Majesty's Stationary Office, London, September 1969. Report of Joint Committee on the use of antibiotics in animal husbandry and veterinary medicine.

288. Swartz MN. 2002. Human diseases caused by foodborne pathogens of animal origin. Clin Infect Dis. 34 Suppl 3:S111-22.

289. Tait-Kamradt A, Davies T, Cronan M, Jacobs MR, Appelbaum PC, Sutcliffe J. 2000. Mutation in 23S rRNA and ribosomal protein L4 account for resistance in pneumococcal strains selected in vitro by macrolide passage. Antimicrob Agents Chemother. 44:2118-2125.

290. Tan SY. 2003. Antoni van Leeuwenhoek (1632-1723): Father of microscopy. Singapore Med J. 44:557-558.

291. Tauch A, Gotker S, Puhler A, Kalinowski J, Thierbach G. 2002. The 27.8-kb R-plasmid pTET3 from *Corynebacterium glutamicum* encodes the aminoglycoside adenyltransferase gene cassette aadA9 and the regulated tetracycline efflux system Tet 33 flanked by active copies of the widespread insertion sequence IS*6100*. Plasmid. 48(2):117-29.

292. Tabaqchali S. 1998. Anaerobic infections in the head and neck region. Scand J Infect Dis. 57: 24-34.

293. Teng LJ, Hsueh PR, Ho SW, Luh KT. 2001. High prevalence of inducible erythromycin resistance among *Streptococcus bovis* isolates in Taiwan. Antimicrob Agents Chemother. 45(12):3362-5.

294. Teng LJ, Hsueh PR, Chen YC, Ho SW, Luh KT. 1998. Antimicrobial susceptibility of viridans group streptococci in Taiwan with an emphasis on the high rates of resistance to penicillin and macrolides in *Streptococcus oralis*. J. Antimicrob. Chemother. 41:621-627.

295. Tenover FC, Weigel LM, Appelbaum PC, McDougal LK, Chaitram J, McAllister S, Clark N, Killgore G, O'Hara CM, Jevitt L, Patel JB, Bozdogan B. 2004. Vancomycin-resistant *Staphylococcus aureus* isolate from a patient in Pennsylvania. Antimicrob Agents Chemother. 48(1):275-80.

296. Tenover FC, McGowan JE Jr. 1996. Reasons for the emergence of antibiotic resistance. Am J Med Sci. 311(1):9-16.

297. Thornsberry C, Ogilvie P, Kahn J, Mauriz Y. 1997. Surveillance of antimicrobial resistance in *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* in the United States in 1996-1997 respiratory season. The Laboratory Investigator Group. Diagn Microbiol Infect Dis. 29(4):249-57.

298. Threlfall EJ, Fisher IS, Berghold C, Gerner-Smidt P, Tschape H, Cormican M, Luzzi I, Schnieder F, Wannet W, Machado J, Edwards G. 2003. Trends in antimicrobial drug resistance in *Salmonella enterica* serotypes Typhi and Paratyphi A isolated in Europe, 1999-2001. Int J Antimicrob Agents. 22(5):487-91.

299. Threlfall EJ, Frost JA, Rowe B. 1999. Fluoroquinolone resistance in salmonellas and campylobacters from humans. BMJ. 318(7188):943-4.

300. Toussaint A, Merlin C. 2002. Mobile elements as a combination of functional modules. Plasmid 47:26-35.

301. Udo EE, Grubb WB. 1996. A phage-mediated transfer of chromosomally integrated tetracycline resistance plasmid in *Staphylococcus aureus*. Curr Microbiol. 32(5):286-90.

302. Van Bambeke F, Balzi E, Tulkens PM. 2000. Antibiotic efflux pumps. Biochem Pharmacol. 60(4):457-70.

303. Van de Beek D, Hensen EF, Spanjaard L, de Gans J, Enting RH, Dankert J. 1997. Meropenem susceptibility of *Neisseria meningitidis* and *Streptococcus pneumoniae* from meningitis patients in The Netherlands. J Antimicrob Chemother. 40:895-897.

304. Van den Bogaard AE, London N, Driessen C, Stobberingh EE. 2001. Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. J Antimicrob Chemother. 47(6):763-71.

305. Van den Bogaard AE, Stobberingh EE. 2000. Epidemiology of resistance to antibiotics. Links between animals and humans. Int J Antimicrob Agents. 14(4):327-35.

306. Van den Braak N, van Belkum A, van Keulen M, Vliegenthart J, Verbrugh HA, Endtz HP. 1998. Molecular characterization of vancomycin-resistant enterococci from hospitalized patients and poultry products in The Netherlands. J Clin Microbiol. 36(7):1927-32.

307. Van Winkelhoff AJ, Herrera Gonzales D, Winkel EG, Dellemijn-Kippuw N, Vandenbroucke-Grauls CM, Sanz M. 2000. Antimicrobial resistance in the subgingival microflora in patients with adult periodontitis. A comparison between The Netherlands and Spain. J Clin Periodontol. 27(2):79-86.

308. Vester B, Douthwaite S. 2001. Macrolide resistance conferred by base substitutions in 23S rRNA. Antimicrob Agents Chemother. 45(1):1-12.

309. Vidaver AK. 2002. Uses of antimicrobials in plant agriculture. Clin Infect Dis. 34 Suppl 3:S107-10.

310. Wade W. 2002. Unculturable bacteria – the uncharacterized organisms that cause oral infections. J R Soc Med. 95:81-83.

311. Walker CB. 2000. The acquisition of antibiotic resistance in the periodontal microflora. Periodontol.10:79-88.

312. Walters MC, Roe F, Bugnicourt A, Franklin MJ, Stewart PS. 2003. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. Antimicrob Agents Chemother. 47(1):317-23.

313. Wang A, Macrina FL. 1995. Streptococcal plasmid pIP501 has a functional *ori*T site. J Bacteriol. 177(15):4199-206.

314. Wang BY, Chi B, Kuramitsu HK. 2002. Genetic exchange between *Treponema denticola* and *Streptococcus gordonii* in biofilms. Oral Microbiol Immunol. 17(2):108-12.

315. Wang Y, Taylor DE. 1991. A DNA sequence upstream of the *tet*(O) gene is required for full expression of tetracycline resistance. Antimicrob. Agents Chemother. 35:2020–2025.

316. Wassenaar TM. http://www.bacteriamuseum.org/aboutus.shtml.

317. Weber P, Filipecki J, Bingen E, Fitoussi F, Goldfarb G, Chauvin JP, Reitz C, Portier H. 2001. Genetic and phenotypic characterization of macrolide resistance in group A streptococci isolated from adults with pharyngo-tonsillitis in France. J Antimicrob Chemother. 48(2):291-4.

318. Wegener HC, Aarestrup FM, Jensen LB, Hammerum AM, Bager F. 1999. Use of antimicrobial growth promoters in food animals and *Enterococcus faecium* resistance to therapeutic antimicrobial drugs in Europe. Em Inf Dis. 5(3):329-335.

319. Weisblum B. 1995. Erythromycin resistance by ribosome modification. Antimicrob Agents Chemother. 39:577-585.

320. Werner G, Klare I, Witte W. 1997. Arrangement of the *vanA* gene cluster in enterococci of different ecological origin. FEMS Microbiol Lett. 155(1):55-61.

321. Whittaker CJ, Klier CM, Kolenbrander PE. 1996. Mechanisms of adhesion by oral bacteria. Annu Rev Microbiol.50:513-52.

322. Whittle G, Whitehead TR, Hamburger N, Shoemaker NB, Cotta MA, Salyers AA. 2003. Identification of a new ribosomal protection type of tetracycline resistance gene, *tet*(36), from swine manure pits. Appl Environ Microbiol. 69(7):4151-8.

323. Whittle G, Shoemaker NB, Salyers AA. 2002. The role of *Bacteroides* conjugative transposons in the dissemination of antibiotic resistance genes. Cell Mol Life Sci. 59(12):2044-54.

324. Whittle G, Hund BD, Shoemaker NB, Salyers AA. 2001. Characterization of the 13-kilobase *ermF* region of the Bacteroides conjugative transposon CTnDOT. Appl Environ Microbiol. 67(8):3488-95.

325. WHO Overcoming Antimicrobial Resistance. World Health Report on Infectious Diseases 2000 Dr Gro Harlem Brundtland Director-General, WHO.

326. Wilson M. 2005. Microbial inhabitants of humans; their ecology and role in health and disease. Cambridge University Press.

327. Wilson M, Harding SD, Dickinson C, Howlett JA, Hobkirk JA. 1990. Cultivable microflora of denture plaque from patients with healthy oral mucosa. Med Sci Res. 18:159-160.

328. Wise R, Hart T, Cars O, Streulens M, Helmuth R, Huovinen P, Sprenger M. 1998. Antimicrobial resistance is a major threat to public health. BMJ. 317(7159):609-10.

329. Witte W. 1994. Medical consequence of antibiotic use in agriculture. Science 279:996-997.

330. Wondrack L, Massa M, Yang BV, Sutcliffe J. 1996. Clinical strain of *Staphylococcus aureus* inactivates and causes efflux of macrolides. Antimicrob Agents Chemother. 40(4):992-8.

331. Woodford N, Warner M, Aucken HM. 2000. Vancomycin resistance among epidemic strains of methicillin-resistant *Staphylococcus aureus* in England and Wales. J Antimicrob Chemother. 45(2):258-9.

332. Woodford N, Johnson AP, Morrison D, Speller DCE. 1995. Current perspectives on glycopeptide resistance. Clin Microbial Rev. 8(4):585-615.

333. Yan JJ, Wu HM, Huang AH, Fu HM, Lee CT, Wu JJ. 2000. Prevalence of polyclonal mefA-containing isolates among erythromycin-resistant group A streptococci in southern Taiwan. J Clin Microbiol. 38:2475-2479.

334. Zarantonelli L, Borthagaray G, Lee EH, Shafer WM. 1999. Decreased azithromycin susceptibility of *Neisseria gonorrhoeae* due to mtrR mutations. Antimicrob Agents Chemother. 43(10):2468-72.

335. Zhanel GG. 43rd Annual ICAAC, Chicago, Symposium. Macrolides: is there a difference? (A microbiologist/pharmacologist perspective).

336. Zhao S, Qaiyumi S, Friedman S, Singh R, Foley SL, White DG, McDermott PF, Donkar T, Bolin C, Munro S, Baron EJ, Walker RD. 2003. Characterization

of *Salmonella enterica* serotype newport isolated from humans and food animals. J Clin Microbiol. 41(12):5366-71.

337. Zheng Z, Stewart PS. 2002. Penetration of rifampin through *Staphylococcus epidermidis* biofilms. Antimicrob Agents Chemother. 46(3):900-3.

338. Ziha-Zarifi I, Llanes C, Kohler T, Pechere JC, Plesiat P. 1999. In vivo emergence of multidrug-resistant mutants of *Pseudomonas aeruginosa* overexpressing the active efflux system MexA-MexB-OprM. Antimicrob Agents Chemother. 43(2):287-91.

339. Zilhao R, Papadopoulou B, Courvalin P. 1988. Occurrence of the Campylobacter resistance gene *tet*(O) in *Enterococcus* and *Streptococcus* spp. Antimicrob Agents Chemother. 32(12):1793-6.

340. Zolezzi P, Laplana LM, Calvo CR, Cepero PG, Erazo MC, Gomez-Lus R. 2004. Molecular basis of resistance to macrolides and other antibiotics in commensal viridans group streptococci and Gemella spp. and transfer of resistance genes to Streptococcus pneumoniae. Antimicrob Agents Chemother. 48(9):3462-7.

Appendix 1: Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristics/source	Ref
Strains		
B. subtilis BS34A	B. *subtilis* CU2189::Tn*916*[a]	241
S. pyogenes AC1	*erm*(B), plasmid pAC1	284
S. pyogenes 02C1064	*mef*(A)	284
S. aureus RN1389	*erm*(A), Tn*554* in the chromosome	284
S. aureus RN4220	*erm*(C), plasmid pE194	284
S. aureus RN4220	*msr*A, plasmid pAT10	284
E. coli BM694 (pAT63)	*ereA*, pBR322 plasmid with cloned insert	284
E. coli BM694 (pAT72)	*ereB*, pUC8 plasmid with cloned insert	284
E. coli L441D	*mphA*	284
E. coli V831	*erm*(F), pVA831 (pBF4) in pBR325 vector	48
E. coli pSL18	*tet*(A)	195
E. coli pRT11	*tet*(B)	195
E. coli pBR322	*tet*(C)	195
E. coli pSL106	*tet*(D)	195
E. coli pSL1504	*tet*(E)	195
E. coli pVB.A15	*tet*(L)	195
E. coli pAT451	*tet*(S)	195
E. coli pNFD13-2	*tet*(Q)	195
Plasmids		
pAM120	pGL101 carrying *Eco*R1 F'(F::Tn*916*) of pAD1	96
pPPM70	pUC18 containing IntronΔkan	242
pGEM-tetW	pGEM carrying a 2.4-kb PCR product with the *tet*(W) gene from B. *fibrisolvens*	7
pGEM-tetO	pGEM carrying the *tet*(O) gene from B. *fibrisolvens*	7
pAT451	pUC18 carrying a 4.5-kb *Cla*I fragment of pIP811 with the *tet*(S) gene	7
pAT102	*tet*(K)	195

[a] This strain originally contained one copy of Tn*916* but during the study was shown to contain two copies of Tn*916*

Appendix 2: Recipes

Buffers, broths, agar	Composition (per litre)
LB (Luria-Bertani) medium and plates	1.5% Tryptone [b] 0.5% Yeast extract [b] 1.0% NaCl [a] (5.0% Technical Agar 3) [b]
SOC medium	2% Tryptone [b] 0.5% Yeast Extract [b] 0.05% NaCl [a] 2.5 mM KCl [a] 10 mM $MgCl_2$ [a]
50 x ETA	2M Tris base [a] 57.1 ml Acetic acid [a] 0.05 M EDTA [a] add distilled H_2O to 1 litre and adjust pH to 8.5 [c]
Hybridization solutions	
Depurination	250 mM HCl [a]
Denaturation	1.5 M NaCl [a] 0.5 M NaOH [a]
Neutralization	1.5 M NaCl [a] 0.5 M TrisHCl [a] pH adjusted to 7.5 [c]
SSC x 20	0.3 M Na_3 citrate [a] 3.0 M NaCl [a] pH 7.0 [c]
Primary wash buffer	0.4% SDS [a] 0.5 x SSC

	Urea

[a] from Sigma

[b] from Oxoid

[c] the pH meter used was from Orion model 520A

Appendix 3: List of the primer sequences used throughout this study

Primer name :	Primer sequences	Size of PCR products in bp	Ref
16S rRNA gene			
27F	5'-AGAGTTTGATCMTGGCTCAG-3'	1465	145
357F	5'-CTCCTACGGGAGGCAGCAG-3'	1135	
1492R	5'-TACGGYTACCTTGTTACGACTT-3'		
M13F	5'-CCCAGTCACGACGTTGTAAAACG-3'		
M13R	5'-AGCGGATAACAATTTCACACAGG-3'		
tet(M)	5'-GTG GAC AAA GGT ACA ACG AG-3'	406	195
	5'-CGG TAA AGT TCG TCA CAC AC-3'		
tet(O)	5'-AAC TTA GGC ATT CTG GCT CAC-3'	515	195
	5'-TCC CAC TGT TCC ATA TCG TCA-3'		
tet(S)	5'-CAT AGA CAA GCC GTT GAC C-3'	667	195
	5'-ATG TTT TTG GAA CGC CAG AG-3'		
tet(W)	5'-GAGAGCCTGCTATATGCCAGC-3'	168	7
	5'-GGGCGTATCCACAATGTTAAC-3'		
tet(K)	5'-TCG ATA GGA ACA GCA GTA-3'	169	195
	5'-CAG CAG ATC CTA CTC CTT-3'		
tet(L)	5'-TCG TTA GCG TGC TGT CAT TC-3'	267	195
	5'-GTA TCC CAC CAA TGT AGC CG-3'		
tet(A)	5'-GCT ACA TCC TGC TTG CCT TC-3'	210	195
	5'-CAT AGA TCG CCG TGA AGA GG-3'		

tet(B)	5'-TTG GTT AGG GGC AAG TTT TG-3' 5'-GTA ATG GGC CAA TAA CAC CG-3'	659	195
tet(C)	5'-CTT GAG AGC CTT CAA CCC AG-3' 5'-ATG GTC GTC ATC TAC CTG CC-3'	418	195
tet(E)	5'-AAA CCA CAT CCT CCA TAC GC-3' 5'-AAA TAG GCC ACA ACC GTC AG-3'	278	195
tet(T)	5'-AAGGTTTATTATATAAAAGTG-3' 5'-AGGTGTATCTATGATATTTAC-3'	169	7
int/xis	5'-CGCCAAAGGATCCTGTATATG-3' 5'-GCTGTAGGTTTTATCAGCTTTTGC-3'	~900	242
ermA	5'-ACGATATTCACGGTTTACCCACTTA-3' 5'-AACCAGAAAAACCCTAAAGACACG-3'	610	193
ermB	5'-TAACGACGAAACTGGCTAAAAT-3' 5'-ATCTGTGGTATGGCGGGTAAG-3'	415	193
ermC	5'-AGTACAGAGGTGTAATTTCG-3' 5'-AATTCCTGCATGTTTTAAGG-3'	520	193
mrsA/B	5'-GCAAATGGTGTAGGTAAGACAACT-3' 5'-ATCATGTGATGTAAACAAAAT-3'	399	193
ermF	5'- CGGGTCAGCACTTTACTATTG-3' 5'-GGACCTACCTCTATGACAAG-3'	466	48
ereA	5'AACACCCTGAACCCAAGGGACG-3' 5' CTTCACATCCGGATTCGCTCGA-3'	420	284
ereB	5'-AGAAATGGAGGTTCATACTTACCA-3' 5'-CATATAATCATCACCAATGGCA-3'	546	284
mphA	5'-AACTGTACGCACTTGC-3'	837	284

	5'-GGTACTCTTCGTTACC-3'		
mef	5'-AGTATCATTAATCACTAGTGC-3'	390	284
	5'-TTCTTCTGGTACTAAAAGTGG-3'		
Bf	5'- TTGGGGCTGTAAAGGGAGGAC- 3'	1900	30
Br	5'- CATCGGTGCTCCATAAC- 3		
Af	5'- GGTACTTGCTTTCCTAAAACTG- 3'	2200	
Set1	5'-CTGTGCCACTGGAAGGAAGT-3'	806	this
	5'-CGGTAGCGATACCCGTTG-3'		study
Set2	5'-CAGTGGGGTCAACAAGATCC-3'	1078	this
	5'-AGTGGGGGTTCCTCCTGTT-3'		study
Set3	5'-CGCTAATCTCATGTCCGTCA-3'	1498	this
	5'-GAAATGGCGTGCAGAAAAAC-3'		study
Set4	5'-TCTGTTCTGCTGTGCCATTC-3'	1250	this
	5'-GCTATTGAATCTGCCCGGTA-3'		study
Set5	5'-CAACGAGTATGCAGGTCGATT-3'	2161	this
	5'-GCTCCAGCATCCTCTGGAC-3'		study

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Prevalence of Tetracycline Resistance Genes in Oral Bacteria

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Genetic Basis of Erythromycin Resistance in Oral Bacteria

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Novel Tetracycline Resistance Determinant from the Oral Metagenome

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